

**Quantifying EBV-specific CD8⁺ T cell immunity in
patients with post transplant lymphoproliferative
disease (PTLD) and other EBV-driven malignancies**

Amy Elizabeth Guppy

Department of Clinical Immunology,
Royal Free and University College Medical School,
University of London

Submitted for the degree of
Doctor of Medicine of the University of London

May 2007



UMI Number: U591248

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U591248

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I confirm that the work presented in this thesis is my own.

Amy Elizabeth Guppy

May 2007

Abstract

Epstein-Barr virus (EBV) specific T cells control EBV-driven proliferation of infected B cells *in vivo*. Deficient EBV immunity predisposes to post-transplant lymphoproliferative disease (PTLD) and EBV-positive Hodgkin's disease (HD). Treatment for PTLD is reduction in immunosuppression (RIS) allowing EBV immune recovery. Current methods of monitoring RIS include immunophenotyping for increases in MHC Class II⁺CD8⁺ T cells, but this is not a specific measure of EBV immunity. A method was developed for enumerating functional EBV-specific T cells using an intracellular cytokine staining (ICS) assay to measure interferon- γ (IFN- γ) production after brief *in vitro* stimulation with EBV antigens.

Analysis of EBV-positive PTLD patients showed a temporal relationship between emergence of EBV-specific IFN- γ ⁺CD8⁺ T cells and PTLD regression in response to RIS. The increase paralleled the rise in MHC class II⁺CD8⁺ T cells. Analysis of tumour infiltrating lymphocytes (TILs) from EBV-positive HD patients showed presence of EBV-specific IFN- γ ⁺CD4⁺ and IFN- γ ⁺CD8⁺ T cells. Although this result indicates EBV-positive HD is not a consequence of deficient EBV immunity, the observed accumulation of CD4⁺ T cells may represent immune suppressive cells.

The ICS assay was demonstrated as a rapid and reliable method for detecting functional EBV immunity, but requirement for several million PBMCs can limit utility. Quantitative RT-PCR (qRT-PCR) was used to measure IFN- γ gene transcription by T cells stimulated with EBV antigen and showed comparable results to ICS using fewer cells.

Clinical assessment of EBV status of tumours is hampered by lack of a single monoclonal antibody able to detect all EBV antigens. A novel monoclonal antibody, RFD3, was used to stain EBV-positive tissue and detected all forms of EBV latency. Results correlated with current diagnostic techniques..

In summary, this thesis describes development and evaluation of novel approaches for detecting EBV and measuring EBV-specific T cell immunity that have potential for clinical and research applications.

Acknowledgements

This work would not have been made possible without the enduring guidance, encouragement, support and friendship from my supervisors; Dr Peter Amlot and Dr Linda Barber. Despite both moving on in their lives since agreeing to be my supervisors, they have remained faithful to my cause for which I will always be very grateful.

I would also like to thank four more good friends who I met during the course of this research; Eira, Fari, Tzveta and Katie. All four have contributed to this thesis in many ways but also made my time in research great fun.

I am indebted to the Special Trustees of the Royal Free Hospital and the Marc Fisher Trust who generously provided me with financial support to conduct this research and also to Professor Alejandro Madrigal for happily agreeing my transfer to the Anthony Nolan Trust in the final years of my research.

I would also like to thank Dr P Mason, Dr J Cavenagh, Dr V Raman, Dr JC Mason, Dr R Banks and all the specialist transplant nurses and patients from the renal transplant centres who provided me with samples and medical information. Without their contribution this research would not have been possible and I have been amazed at the help and encouragement we have received from them all. My thanks also go to Dr Ann-Margaret Little for providing all the HLA typing for all my samples.

My final thanks are for my wonderful family. Their constant love and belief in me made the dark times easier to endure and the good times cause for celebration. Without them this thesis would simply never have been finished. In particular, my special thanks are for my husband and best friend, Alan, whose love for me and our beautiful children is endless. It is to them that I dedicate this work.

Abbreviations

ABVD	Doxorubicin, Bleomycin, Vinblastine, Dacarbazine
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
Aza	Azathioprine
BART	<i>Bam</i> HIA rightward transcripts
BCR	B cell receptor
BL	Burkitt's lymphoma
bp	base pair
BSA	Bovine serum albumin
CFT	Complement fixation staining
CLIP	Class II-associated invariant chain peptide
CM	Complete media
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CNS	Central nervous system
CR	Complete response
CSA	Cylosporin A
CSF	Colony stimulating factor
CTL	Cytotoxic T cell
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DoD	Died of Disease
EBER	EBV-encoded RNAs
EBNA	EBV nuclear antigens
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
FACs	Fluorescent Activated Cells sorter
FFP	Freedom from progression
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GVHD	Graft versus Host disease
HD	Hodgkin's Disease
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HSCT	Haematopoietic stem cell transplant
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis

IP staining	Immunoperoxidase staining
ISH	<i>In situ</i> hybridisation
LCL	Lymphoblastoid cell line
LDA	Limiting dilution assay
LDH	Lactate dehydrogenase
LMP	Latent membrane protein
LN	Lymph node
LTF	Lost to follow-up
MHC	Major histocompatibility
MMF	Mycophenolate mofetil
ML-B	Malignant lymphoma B cell type
M-MLV	Murine Moloney Leukaemia Virus
MRD	Minimal residual disease
NHL	non-Hodgkin's lymphoma
NK cell	Natural killer cell
NPC	Nasopharyngeal carcinoma
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Progressive disease
PE	Phytoerythrin
PHA	Phytohaemagglutinin
PTLD	Post transplant lymphoproliferative disease
qRT-PCR	Quantitative real-time PCR
rb gene	retinoblastoma gene
RIS	Reduction in immunosuppression
RNA	Ribonucleic acid
R-S cell	Reed-Sternberg cell
RT	Reverse transcriptase
SAP	SLAM-associated protein
SCID	Severe combine immunodeficiency
sd	Standard deviation
SD	Stable disease
SHM	Somatic hypermutation
SLAM	Signalling lymphocytic activation molecule
SOT	Solid organ transplant
SSC	Side scatter
TAC	Tacrolimus
<i>taq</i>	<i>Thermus aquaticus</i>
TCR	T cell receptor
TIL	Tumour infiltrating lymphocyte
TNF	Tumour necrosis factor
TRM	Treatment related mortality
XLP	X-linked lymphoproliferative disease

Table of Contents

Quantifying EBV-specific CD8⁺ T cell immunity in patients with post transplant lymphoproliferative disease (PTLD) and other EBV-driven malignancies.....	1
Abstract	3
Acknowledgements.....	4
Abbreviations.....	5
Table of Contents	7
List of Tables.....	13
List of Figures	15
Chapter 1 Introduction	18
<i>1.1 Epstein-Barr virus</i>	<i>19</i>
1.1.1 The molecular Biology of EBV	20
1.1.2 Patterns of EBV gene expression	25
1.1.3 EBV epidemiology	26
1.1.4 EBV infection of B cells.....	27
<i>1.2 Immune responses to EBV and evasion strategies employed by the virus.....</i>	<i>31</i>
1.2.1 General immune responses to viruses.....	31
1.2.2 Innate response to EBV	36
1.2.3 B cell responses to EBV	37
1.2.4 T cell responses to EBV	37
1.2.5 EBV immune evasion strategies.....	42
<i>1.3 EBV associated malignancies: The role of EBV in pathogenesis.....</i>	<i>45</i>
1.3.1 EBV-associated lymphoid malignancies	45

1.3.2	EBV associated epithelial malignancies	55
1.4	<i>Clinical Management of PTLD</i>	58
1.4.1	Incidence and risk factors	59
1.4.2	Clinical features	60
1.4.3	Diagnosis	61
1.4.4	Prognosis	64
1.4.5	Treatment	65
1.5	<i>Clinical management of EBV-positive HD</i>	74
1.5.1	Incidence	74
1.5.2	Clinical presentation	75
1.5.3	Diagnosis	76
1.5.4	Prognosis	76
1.5.5	Treatment	77
1.6	<i>Aims of the thesis</i>	81
Chapter 2 Materials and Methods		82
2.1	<i>Peptide Biochemistry</i>	83
2.1.1	Source	83
2.1.2	EBV specific peptides	83
2.2	<i>In vitro Cell Culture</i>	85
2.2.1	Blood sample collection	85
2.2.2	Media and Reagents	86
2.2.3	Cell culture conditions	86
2.2.4	Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood	87
2.2.5	Whole blood preparation	87
2.2.6	Single cell suspension from lymph node biopsies	88
2.2.7	Cell counting and viability	88
2.2.8	Cell cryopreservation and thawing	88

2.2.9	Generation of EBV-transformed B lymphoblastoid cell lines	89
2.2.10	Characteristics of EBV-transformed B lymphoblastoid cell lines	89
2.2.11	EBV specific T cell stimulation using LCLs or HLA-restricted EBV peptides.....	90
2.2.12	Tumour cell lines used for immunohistology	93
2.2.13	<i>Mycoplasma</i> testing and treatment.....	93
2.3	<i>Cellular assays</i>	95
2.3.1	Immunofluorescent antibody staining for cell surface antigens	95
2.3.2	Immunofluorescent antibody staining for intracellular antigens	96
2.3.3	Immunofluorescent antibodies	97
2.3.4	Multi-parameter flow cytometry.....	100
2.3.5	Statistical analyses	100
2.4	<i>Molecular Biology</i>	101
2.4.1	RNA extraction.....	101
2.4.2	Generation of complementary DNA.....	102
2.4.3	Gene amplification by Polymerase chain reaction	103
2.4.4	DNA electrophoresis	104
2.4.5	Quantitative real-time PCR (RT-PCR)	106
2.4.6	The comparative Ct method ($\Delta\Delta Ct$) for relative quantification of gene expression.....	109
2.5	<i>Immunohistology</i>	110
2.5.1	Preparation of Cytospins	110
2.5.2	Preparation of fresh tissue sections.....	111
2.5.3	Preparation of formalin-fixed tissue sections	111
2.5.4	Antibodies used to detect EBV antigen	111
2.5.5	Detection of EBV by immunoperoxidase staining	112
2.5.6	Detection of EBV by complement indirect immunofluorescent technique	113
2.5.7	Detection of Latent EBV infection by <i>in situ</i> hybridisation	114

Chapter 3 Development of a rapid immuno-assay to enumerate EBV-specific T cell responses:

Healthy Individuals.....	115
3.1 <i>INTRODUCTION</i>	116
3.2 <i>RESULTS</i>	124
3.2.1 Quantification of EBV-specific T cell responses using EBV-transformed LCLs as stimulators presenting EBV antigens.....	124
3.3 <i>DISCUSSION</i>	152

Chapter 4 Development of a rapid immunoassay to measure EBV-specific T cell responses:

Patients with EBV-driven malignancies.....	159
4.1 <i>INTRODUCTION</i>	160
4.2 <i>RESULTS</i>	165
4.2.1 Measurement of EBV-specific CD8 ⁺ T cell responses in patients with PTLD.....	165
4.2.2 Assessment of EBV-specific T cell responses in patients with EBV-positive Hodgkin's Disease.....	189
4.3 <i>DISCUSSION</i>	204

Chapter 5 Assessment of quantitative real-time polymerase chain reaction (qRT-PCR) to measure IFN-γ production by EBV-specific T cells using small cell numbers.....

5.1 <i>INTRODUCTION</i>	213
5.2 <i>RESULTS</i>	220
5.2.1 Assessment of a qRT-PCR assay to detect and quantify IFN-γ production by EBV- specific T cells from limited cell numbers.....	220
5.2.2 Comparison of ICS and qRT-PCR for measuring IFN-γ production from EBV-specific T cells after stimulation with peptides	224
5.2.3 Comparison of ICS and qRT-PCR for measuring IFN-γ production from EBV-specific T cells after stimulation with LCLs.....	227

5.2.4	Comparison of ICS and qRT-PCR for measuring IFN- γ production by EBV-specific T cells in patients with PTLD	232
5.3	<i>DISCUSSION</i>	236
Chapter 6 Detection of all patterns of EBV latency in human cells and tissues using a novel monoclonal antibody, RFD3.....		240
6.1	<i>INTRODUCTION</i>	241
6.2	<i>RESULTS</i>	243
6.2.1	Nuclear staining of fresh frozen cryostat sections of lymph node and tumour tissues by RFD3.....	243
6.2.2	Comparison of the efficacy of RFD3 versus LMP/EBNA-2 (IP) and EBNA CFT staining, using a range of EBV positive and negative cell lines	247
6.2.3	Determining the ability of RFD3 to identify cells infected with other members of the herpes virus family, namely HHV-8 and CMV	251
6.2.4	Attempting to separate staining of EBV positive nuclei and staining of FDCs.....	254
6.3	<i>DISCUSSION</i>	255
Chapter 7 General Conclusions		258
7.1	<i>GENERAL CONCLUSIONS</i>	259
Appendices		266
<i>Appendix 1.1</i>		266
Clinical Staging of Hodgkin's and Non-Hodgkin's Lymphomas (Ann Arbor Classification) ...		266
<i>Appendix 1.2</i>		267
Karnofsky Scale.....		267
<i>Appendix 1.3</i>		268
Response evaluation criteria in solid tumours: RECIST criteria		268
<i>Appendix 1.4</i>		269

GAPDH Primers	269
<i>Appendix 1.5</i>	270
Serial measurement of EBV-specific T cell responses in EBV-positive and EBV-negative PTLD patients.....	270
References	277

List of Tables

Table 1.1: Products of EBV during latent infection	24
Table 1.2: Expression of EBV latent genes in disease	25
Table 1.3: Risk factors for developing PTLD	60
Table 1.4: Protocol for RIS	67
Table 2.1: EBV peptide sequences presented by HLA-A	84
Table 2.2: EBV peptide sequences presented by HLA-B and HLA-Cw	85
Table 2.3: Example of a panel of partially HLA-matched LCLs used for one healthy individual	92
Table 2.4: Directly conjugated fluorochrome- labelled antibodies used in this study.....	98
Table 2.5: Antibodies used in the indirect method of staining for the characterisation of cell surface activation markers.....	99
Table 2.6: Reagents used in the synthesis of cDNA from RNA.....	103
Table 2.7: Reagents and the PCR thermal cycler program used to amplify GAPDH by conventional PCR.....	104
Table 2.8: Protocol for quantitative RT-PCR using the TaqMan® gene expression kit	107
Table 2.9: Antibodies used to detect EBV by immunoperoxidase staining	112
Table 3.1: Available assays for enumerating EBV-specific T cells	123
Table 3.2: Summary of results obtained for five healthy EBV sero-positive individuals using single HLA class I allele matched LCLs as stimulators	139
Table 4.1: Characteristics of patients who developed a PTLD following renal transplantation.....	166
Table 4.2: Patient samples.....	168
Table 4.3: Sources of EBV antigen used to stimulate an EBV-specific CD8+ T cell response in each PTLD patient	170
Table 4.4: Characteristics of patients who developed HD.....	190
Table 6.1: RFD3 nuclear staining in reactive, malignant and lymphoma tissues in relation to EBV associated pathologies.....	245

Table 6.2: Percentage of nuclei staining by RFD3, EBER and EBNA CFT in EBV positive pathologies	
.....	246
Table 6.3: RFD3 staining of EBV-positive cell lines compared to other methods.....	250
Table 6.4: RFD3 staining of EBV-negative cell lines compared to other methods.....	251
Table 6.5: RFD3 staining of different Herpes virus positive cell lines compared to other methods	252

List of Figures

Figure 1.1: The Epstein-Barr virus genome	21
Figure 1.2: Proposed models of primary and persistent infection of B cells by EBV	28
Figure 1.3: B cell development	29
Figure 1.4: Antigen presentation via the endogenous and exogenous pathways	34
Figure 1.5: The dynamics of T cell responses to EBV	39
Figure 1.6: Bystander pathogenesis model of Burkitt's lymphoma	47
Figure 1.7: Pathogenesis of EBV-positive HD	50
Figure 1.8: Pathogenesis of EBV-positive PTLD	53
Figure 1.9: Incidence of HD by age	75
Figure 2.1: Schemata to show method of EBV specific T cell stimulation	91
Figure 2.2: Mycoplasma detection	94
Figure 2.3: A representative example of GAPDH amplification by conventional PCR	106
Figure 2.4: The TaqMan® gene expression assay	108
Figure 2.5: A representative spreadsheet of data showing calculations used in the comparative threshold Ct method	110
Figure 3.1: Comparison of cytokine production by EBV-specific T cells	125
Figure 3.2: A representative example of EBV-specific T cell responses after stimulation with autologous LCLs	127
Figure 3.3: Amplification of the EBV-specific T cell response using previously in vitro expanded antigen-specific CD8+ T cells	128
Figure 3.4: Experiments to determine the optimal assay duration	129
Figure 3.5: Comparison of EBV-specific T cell responses detected using PBMCs (A) and red cell lysed whole blood (B) and different ratios of LCLs as stimulators	131
Figure 3.6: Serial monitoring of EBV sero-positive healthy individuals to determining the consistency with which the assay detects EBV-specific T cell responses	133

Figure 3.7: EBV-specific T cell responses after stimulation with single HLA class I allele matched LCLs in five healthy individuals.....	137
Figure 3.8: Optimisation of the ICS assay to enumerate EBV-specific T cell responses in PBMCs stimulated with synthetic peptides	142
Figure 3.9: Example of EBV-specific T cell responses seen on stimulation with individual and pools of HLA-A*02 restricted EBV peptides	143
Figure 3.10: Spectrum of EBV-specific T cell responses to HLA-A*02 restricted EBV peptides in four HLA-A*02 healthy individuals.....	144
Figure 3.11: Example of EBV-specific T cell responses seen on stimulation with pools EBV peptides	148
Figure 3.12: Comparison of EBV-specific T cell responses detected using HLA class I restricted peptide pools in PBMCs from six healthy individuals.....	150
Figure 4.1: Serial measurement of EBV-specific T cell responses in three EBV-positive PTLN patients during RIS.....	176
Figure 4.2: Serial measurement of EBV-specific T cell responses in two representative patients with EBV-negative PTLNs during RIS	180
Figure 4.3: Comparison of peak EBV-specific T cell responses on stimulation with different EBV antigens in patients with PTLN.....	182
Figure 4.4: Representative example from patient EBV ⁺ 3 of EBV-specific T cell responses after stimulation with a panel of single HLA class I allele matched LCLs	184
Figure 4.5: Magnitude of the EBV immune response at initiation of RIS compared to the maximum response detected	188
Figure 4.6: Comparison of CD8 ⁺ T cell responses from patients with EBV-positive HD, EBV-negative HD and Reactive hyperplasia.....	195
Figure 4.7: Comparison of the magnitude of the EBV-specific CD8 ⁺ T cell response and the proportion of CD8 ⁺ T cells expressing activation markers within the LNs of patients with EBV-positive and EBV-negative HD and Reactive Hyperplasia	196
Figure 4.8: Comparison of CD4 ⁺ T cell responses in patients with EBV-positive HD, EBV-negative HD and Reactive hyperplasia.....	199

Figure 4.9: Comparison of the magnitude of the EBV-specific CD4 ⁺ T cell response and the proportion of CD4 ⁺ T cells expressing activation markers within the LNs of patients with EBV-positive and EBV-negative HD and Reactive Hyperplasia	200
Figure 4.10: EBV-specific T cell responses to Autologous LCLs in patients with EBV-positive and EBV-negative HD	201
Figure 5.1: The kinetics of IFN- γ mRNA expression following stimulation with HLA-A*0201-restricted EBV peptides in a healthy HLA-A*0201 individual	222
Figure 5.2: Assessment of the minimum number of PBMCs required to detect IFN- γ mRNA expression following stimulation with HLA-A*0201 restricted EBV peptide pools.....	224
Figure 5.3: Comparison of ICS and qRT-PCR to measure IFN- γ production by EBV-specific T cells using EBV peptides as stimulators	226
Figure 5.4: Comparison of ICS and qRT-PCR to measure IFN- γ production by EBV-specific T cells using EBV-transformed LCLs as stimulators	229
Figure 5.5: Comparison of IFN- γ mRNA expression detected from EBV-specific T cells using different numbers of LCLs as stimulators	231
Figure 5.6: Comparison of ICS with qRT-PCR for measuring EBV-specific T cell immunity in a PTLD patient with high numbers of IFN- γ producing EBV-specific T cells.....	234
Figure 5.7: Comparison of ICS with qRT-PCR for measuring EBV-specific T cell immunity in a PTLD patient with low numbers of IFN- γ producing EBV-specific T cells.....	235
Figure 6.1: RFD3 nuclear staining of EBV-positive tissues.....	244
Figure 6.2: Staining of EBV-positive cell lines with RFD3, EBNA2 and LMP mAbs	249
Figure 6.3: Staining of herpes virus-positive cell lines with RFD3 and LN53 mAbs	253

Chapter 1

Introduction

1.1 Epstein-Barr virus

Epstein-Barr virus (EBV; HHV-4) is a member of the ancient and highly successful γ herpes virus family and is ubiquitous amongst the adult population worldwide. The virus probably evolved from a nonhuman-primate virus as EBV shares sequences and a genetic organization similar to the EBV-like virus of the rhesus monkey and both viruses maintain persistent infection in the oropharynx and B cells (Moghaddam *et al.*, 1997). Through millions of years of co-evolving with its human host, EBV has developed supreme survival strategies to establish a cycle of lifelong latent infection of memory B lymphocytes with intermittent reactivation and productive (lytic) phases, to ensure spread to other susceptible hosts. This process ordinarily has little impact on the health of the majority of the infected population, however the latent genes of EBV have oncogenic potential as shown by their ability to immortalise B lymphocytes (Pope *et al.*, 1968).

EBV was originally discovered in a B cell line cultured from Burkitt's lymphoma cells (Epstein *et al.*, 1964), and is now known to be present in greater than 95% of all African Burkitt's lymphomas (Magrath, 1990). Subsequent studies have shown that EBV is also associated with a variety of other human tumours including Hodgkin's disease (HD) (Weiss *et al.*, 1989), post transplant lymphoproliferative disease (PTLD) (Crawford *et al.*, 1980), nasal T/ natural killer (NK) cell lymphoma (Jones *et al.*, 1988; Kanavaros *et al.*, 1993; Tsuchiyama *et al.*, 1998), leiomyosarcoma in immunocompromised patients (McClain *et al.*, 1995) and epithelial cancers such as nasopharyngeal carcinoma (zur Hausen *et al.*, 1970) and gastric carcinoma (Rowlands *et al.*, 1993; Shibata and Weiss, 1992).

All of these tumours are characterised by the presence of extrachromosomal copies of the circular viral genome in the cancer cells and the expression of the EBV-encoded latent genes, which appear to contribute to the malignant phenotype (Rickinson and Kieff, 2001).

1.1.1 The molecular Biology of EBV

The EBV genome was the first of the herpes viruses to be completely cloned and sequenced (Baer *et al.*, 1984) and is composed of linear double-stranded deoxyribonucleic acid (DNA) of approximately 172 kilobase pairs (kb) in length. The genome exists in linear form in viral particles, however, after infection of B cells, the virus circularises and persists as an episome in the nucleus (**Figure 1.1**), thereby establishing a latent infection. Periodically, the virus reactivates, switching to a lytic cycle where it can proliferate and release new viral particles. Latently infected B cells express a range of EBV-encoded proteins consisting of the EBV nuclear antigens, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP and the latent membrane proteins LMP1, LMP2A and LMP2B. The pattern of expression of these latent proteins differs between normal and malignant B cells and is associated with different types of EBV-malignancies (as discussed later). In addition to the latent proteins, two types of EBV-encoded non-translated ribonucleic acid (RNA) are also transcribed in latency; EBV-encoded RNAs (EBERs) and *Bam*HIA rightward transcripts (BARTs). Each of these products of EBV infection are intrinsically associated with oncogenesis by interacting with, or exhibiting homology with, a variety of anti-apoptotic molecules, cytokines and signal transducers and are further described below and in **Table 1.1**.

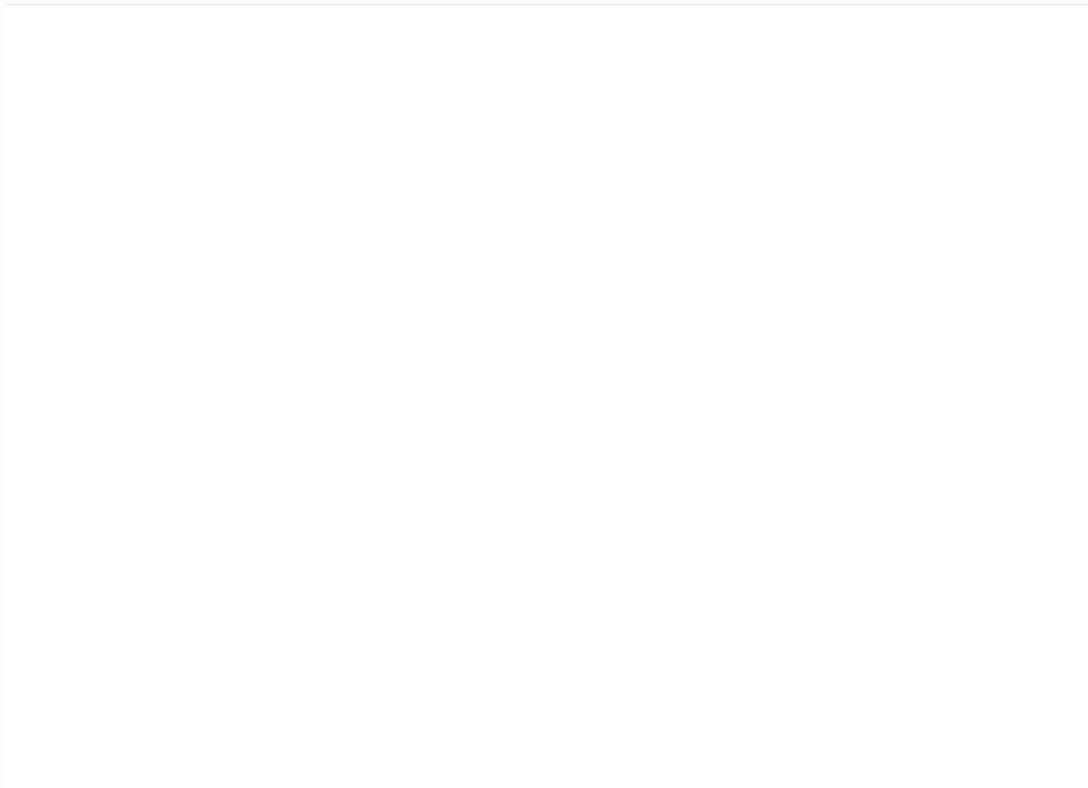


Figure 1.1: The Epstein-Barr virus genome

Viral replication proceeds from the origin of plasmid replication (oriP) in a clockwise direction. The black arrows indicate the position of the coding exons for each of the latent proteins; the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. LMP2A and LMP2B are composed of multiple exons located either side of the terminal repeat (TR) region, which is formed during the circularisation of the linear DNA to produce the viral episome. The outer red clockwise arrow represents EBV transcription during a form of latency known as latency III where all the EBNAs are transcribed from either the Cp or Wp promoter. The inner blue clockwise arrow represents transcription of EBNA1 which originates from the Qp promoter during latency I and II.

Figure adapted from (Young and Murray, 2003).

EBNA1: EBNA1 is vital to the maintenance and replication of the episomal EBV genome (Yates *et al.*, 1985). It exerts its function by binding to the plasmid origin of viral replication (OriP, **Figure 1.1**) and is able to interact with viral promoters up-

regulating transcription of other EBNA proteins and LMP1. A series of Gly-Ala repeats within its sequence interferes with antigen processing and major histocompatibility (MHC) class I-restricted presentation (Levitskaya *et al.*, 1995), and it was previously thought that EBV-infected cells that express EBNA1 alone were unable to be recognised by EBV-specific CD8⁺ T cells. However a recent study has challenged this and shown that EBNA-1 is able to be presented to CD8⁺ T cells via the MHC class I pathway but reactive cells produce interferon- γ (IFN- γ) rather than lytic activity (Lee *et al.*, 2004)

EBNA2: EBNA2 is one of the first viral proteins to be expressed after infection of B cells *in vitro* and is essential for cell immortalisation (Cohen *et al.*, 1989). It is able to indirectly activate the transcription of both cellular genes such as *c-myc* (a cellular proto-oncogene) and CD23 (a B cell surface activation antigen) as well as LMP1 and LMP2A (Zimber-Strobl and Strobl, 2001) by binding to transcription factors such as the viral Cp binding factor. EBNA2 has been shown to interact with other transcription factors involved in the *Notch* signalling pathway (J κ -recombination-binding protein, RBP-J κ (Grossman *et al.*, 1994; Hsieh and Hayward, 1995)) which has been implicated in oncogenesis (reviewed by (Lefort and Dotto, 2004).

EBNA 3A, 3B and 3C: These gene products are all transcriptional regulators and also able to interact with Cp binding factor 1 involved in the *notch* signalling pathway. EBNA 3A and EBNA 3C are thought to be crucial in the transformation of B cells *in vitro*, whereas EBNA 3B is not. EBNA 3C is thought to achieve this by disrupting the cell-cycle cell points mediated by the *retinoblastoma* (rb) tumour suppressor gene products (Parker *et al.*, 1996).

EBNA-LP: Together with EBNA2, this protein is also produced early after infection of B cells. EBNA-LP interacts with EBNA2 and together bind and inactivate *p53* and *rb*, forcing the resting B cells into the G1 phase of the cell cycle (Szekely *et al.*, 1993). EBNA-LP also interacts with transcription factors involved in the *notch* signalling pathway.

LMP1: LMP1 is the main transforming protein of EBV. It is able to exert its oncogenic effects by acting as a functional homologue of CD40, a receptor on germinal centre B cells. CD40 is also a member of the tumour necrosis factor receptor (TNFR) superfamily and activation via LMP1 or T helper cells results in the expression of B-cell adhesion molecules, B cell activation markers (CD23, CD39, CD44 and MHC class II) (Wang *et al.*, 1990) as well as the anti-apoptotic molecules bcl-2 and A20 (Laherty *et al.*, 1992; Eliopoulos *et al.*, 1997). LMP1 is therefore able to drive B cell proliferation and rescue B cells from apoptosis.

LMP2A and LMP2B: The LMP2 proteins are not essential for EBV transformation of B cells, but are able to promote the latency of the virus in B cells by modifying B cell development. LMP2A contains immunoglobulin tyrosine-based activation motifs (Alber *et al.*, 1993) that are also present in the B cell co-receptors CD79A and CD79B. LMP2A is able to inhibit B cell receptor (BCR) signalling by binding and sequestering tyrosine kinases involved in signalling pathway (Miller *et al.*, 1995). This then prevents unwanted antigen-induced activation of EBV infected B cells that would cause reactivation and entry into the lytic cycle. LMP2A is able to transform epithelial cells *in vitro* (Scholle *et al.*, 2000), and is implicated in the pathogenesis of NPC and gastric carcinoma.

Table 1.1: Products of EBV during latent infection
(Adapted from (Thompson and Kurzrock, 2004).

EBER1 and EBER 2: EBERs are thought to promote persistence of the virus by inhibiting the anti-viral effects of IFNs by binding to the IFN-inducible, double stranded-RNA-dependant protein kinase, PKR (Takada and Nanbo, 2001; Nanbo *et al.*, 2002). EBERs have also been implicated in the suppression of EBV-specific cytotoxic T cells by increasing the secretion of IL-10 thus inhibiting the T_H1 response (Kitagawa *et al.*, 2000; Ruf *et al.*, 2000)

1.1.2 Patterns of EBV gene expression

EBV infected B cells *in vivo* express different patterns of latent gene expression. Memory B cells obtained from healthy individuals express only EBER and LMP2 and possibly EBNA1, however the expression in patients with EBV-associated diseases varies and has been classified in to three latency programmes (**Table 1.2**).

EBV-transformed B cells proliferating *in vitro* express latency programme III (Rickinson and Kieff, 2001).

<i>Latency Programme</i>	<i>EBNA1</i>	<i>EBNA2</i>	<i>EBNA3</i>	<i>LMP1</i>	<i>LMP2</i>	<i>EBER</i>	<i>Disease</i>
Type I	+	-	-	-	-	+	Burkitt's lymphoma PTLD-BL type.
Type II	+	-	-	+	+	+	NPC, HD, peripheral T-cell lymphoma, PTLD-HD type.
Type III	+	+	+	+	+	+	PTLD-DLBC type, X-linked lymphoproliferative disease, Infectious mononucleosis
Type 0	+/-	-	-	-	+/-	+/-	Healthy individuals

Table 1.2: Expression of EBV latent genes in disease

+ = positive expression, - = negative expression, +/- = possible expression, PTLD = post transplant lymphoproliferative disease, BL = Burkitt's lymphoma, HD = Hodgkin's disease, DLBC = Diffuse large B cell.

1.1.3 EBV epidemiology

EBV is unable to cross the placenta and infects individuals from birth onwards, leading to a cumulative incidence of over 90% of the world's population. Infected individuals shed virus either intermittently or continuously into their saliva (Yao *et al.*, 1985; Niederman *et al.*, 1976) and primary infection typically occurs during early childhood from close contact between family members (Gratama *et al.*, 1990; Fleisher *et al.*, 1981). Primary infection in young children is usually a subclinical illness (Fleisher *et al.*, 1979) and occurs at an early age in communities with poor standards of hygiene (Biggar *et al.*, 1978), however, in areas of higher standards of living, primary infection is often delayed until early adulthood where it causes infectious mononucleosis (IM). Early studies showed that IM occurred in 50-74% of young adults (Hallee *et al.*, 1974; Niederman *et al.*, 1970; Sawyer *et al.*, 1971), however, a recent prospective study amongst university students showed that IM developed in only 25% (Crawford *et al.*, 2006).

It is widely assumed that primary infection in adolescence occurs through the saliva shared through kissing, however, the virus has been detected in both female (Sixbey *et al.*, 1986) and male (Israele *et al.*, 1991) genital secretions raising the possibility of sexual transmission. In addition, correlations have been made between both EBV seropositivity and history of IM, with high numbers of sexual partners in university students (Crawford *et al.*, 2002).

EBV can also be transmitted via blood and organ donation through the receipt of latently infected B lymphocytes (Alfieri *et al.*, 1996; Cen *et al.*, 1991). Seronegative

organ recipients from a seropositive donor are estimated to have an 80% risk of primary EBV infection in the first year following transplant (Ho *et al.*, 1988).

1.1.4 EBV infection of B cells

EBV is able to infect B cells through the interaction of the major viral envelope glycoprotein gp350 with the complement receptor CD21 on the surface of B cells (Nemerow *et al.*, 1985). Following this a second envelope glycoprotein, gp42 binds to MHC class II molecules (Li *et al.*, 1997) and the virus is able to enter the cell.

In healthy individuals, EBV infected B cells are confined to the memory compartment however the precise route by which the virus accesses these cells remains controversial. Two mechanisms have been proposed (**Figure 1.2**). The first based on detailed analysis of tonsillar B cells suggest that EBV predominantly infects the naïve B cells and mimics the physiological process of antigen-driven memory cell development in the germinal centres of lymphoid tissues, detailed in **Figure 1.3** (Babcock *et al.*, 2000; Babcock and Thorley-Lawson, 2000; Joseph *et al.*, 2000). This model suggests that EBV switches on a growth programme causing newly infected naïve B cells to proliferate and express all of the latent viral genes. Proliferation is then terminated by EBV down-regulating viral expression to EBNA1, LMP1 and LMP2 (latency programme II). LMP1 and LMP2A ensure selection and survival of EBV-infected B cells in the germinal centres by functioning as a CD40 homologue (LMP1) and by mimicking BCR engagement (LMP2A). Infected B cells then differentiate into memory B cells and exit into the peripheral circulation. This model, however, is unable to explain why immunohistochemical and *in situ* hybridisation studies in tonsils of patients with IM showed localisation of EBV-infected B cells in

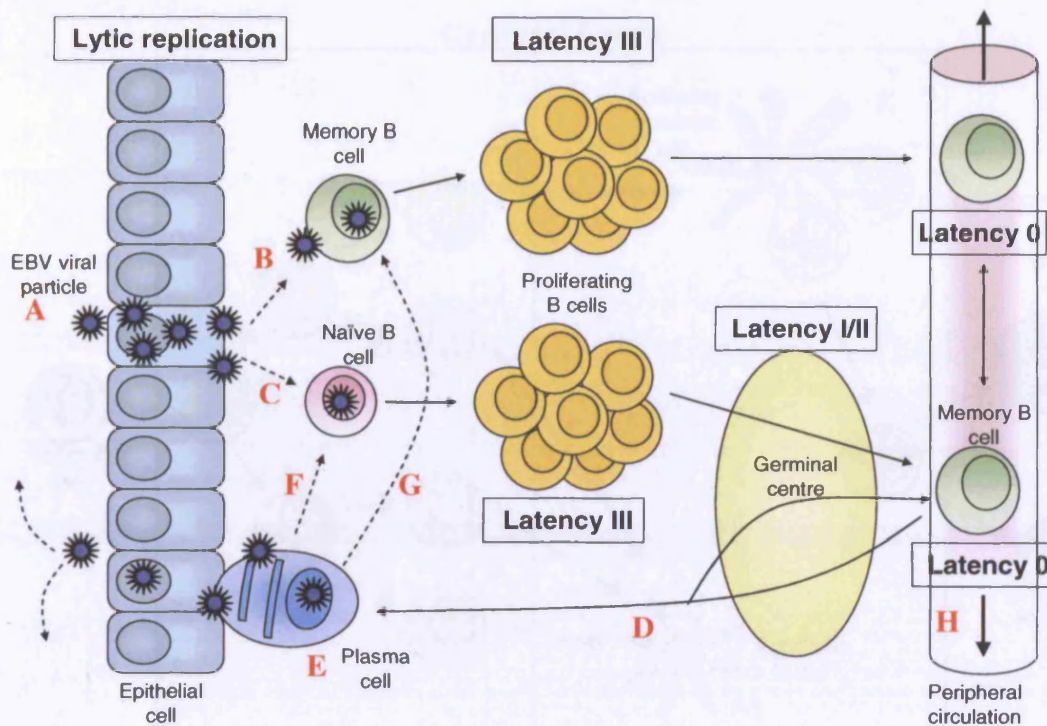



Figure 1.2: Proposed models of primary and persistent infection of B cells by EBV

EBV viral particles establish a primary focus of lytic replication in the oropharynx (A) with possible involvement of the epithelial cells. Infection then spreads to B cells populating the lymphoid tissues. Two models have been proposed for how memory B cells become infected. The first (B) suggests that EBV directly infects the memory B cells population of the lymphoid tissues. EBV switches on viral genes associated with latency III resulting in widespread proliferation. Eventually proliferation is terminated by the down regulation of all or most of the EBV latent genes and memory B cells exit into the peripheral circulation. The second model (C) suggests that EBV predominantly targets naïve B cells population and enters the memory B cell reservoir by exploiting the physiological antigen-induced selection of memory B cells that occurs in the germinal centres (Figure 1.3). Following proliferation of the naïve B cells driven by EBV expressing the latency III programme, EBV down-regulates latent gene expression during transit through the germinal centres before differentiation into memory B cells. Persistent infection (D) and completion of the EBV life cycle occurs during antigen-driven activation of B cells. This recruits memory B cells into the germinal centres where activation of viral latency programmes may occur. Memory B cells may differentiate into plasma cells, with resultant activation of the lytic cycle and production of viral particles (E). These particles can be shed into the saliva and infect a second host. Alternatively viral particles can directly infect the naïve (F) or memory B cells (G). Memory B cells may also exit the germinal centres and re-enter the peripheral circulation, replenishing the EBV reservoir (H).

 = EBV viral particle

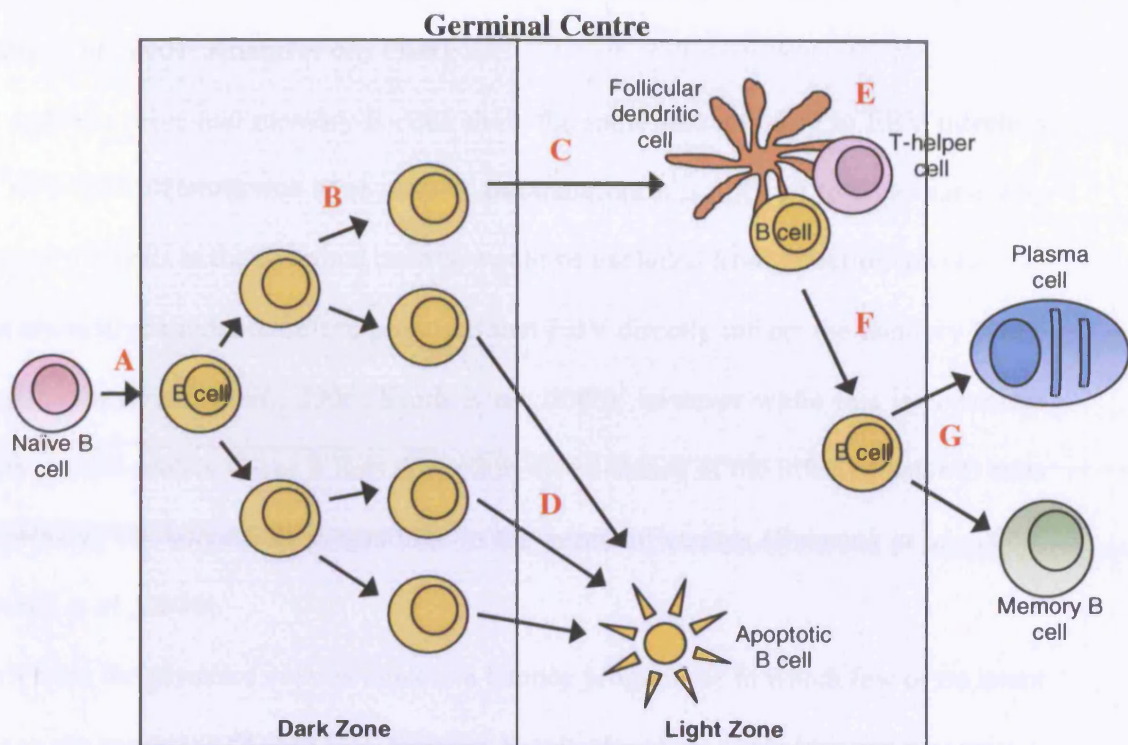


Figure 1.3: B cell development

Naïve B cells expressing a functional BCR are released into the peripheral circulation from the bone marrow. On encountering cognate antigen and activated T-helper cells, B cells become activated and migrate to the dark zone of the germinal centres within secondary lymphoid organs (**A**). The activated B cells then reduce expression of their surface immunoglobulin and undergo rapid cell division and mutation of their rearranged immunoglobulin (Ig) genes (**B**). This process is termed somatic hypermutation and creates B cells with BCRs of different affinities for a particular antigen. B cell proliferation eventually stops and surface expression of Ig is increased. B cells then migrate to the light zone of the germinal centre where they must interact with follicular dendritic cells and T-helper cells in order to survive. B cells compete for binding of the antigen presented by the follicular dendritic cells and are selected according to the binding affinity of the membrane Ig (**C**). B cells expressing the high-affinity Ig pass this selection process and receive a second survival signal from the T-helper cells via the CD40/CD40 ligand interaction (**E**). This prompts the selected B cells to undergo further repeated rounds of proliferation. At this stage the immunoglobulin genes of many of the selected B cells are remodelled by class-switching (**F**). This process alters the Ig heavy-chain constant region resulting in a switch of Ig isotype from IgM/IgD to IgG, IgA or IgE without altering antigen specificity. Selected germinal centre B cells then differentiate into memory B cells or plasma cells, leave the germinal centres and enter the peripheral circulation (**G**). B cells expressing Ig with lower binding affinities fail to pass the selection process and die by apoptosis (**D**).

the extra-follicular areas and rarely in the germinal centres (Niedobitek *et al.*, 1992; Meru *et al.*, 2001; Araujo *et al.*, 1999).

In addition naïve and memory B cells show the same susceptibility to EBV infection *in vitro* (Ehlin-Henriksson *et al.*, 2003), and therefore it is difficult to understand why memory B cells in the germinal centres would be excluded from infection *in vivo*.

An alternative model therefore proposed that EBV directly infects the memory B cell population (Kurth *et al.*, 2000; Kurth *et al.*, 2003), however while this is consistent with the IM studies above it fails to explain the existence of the infected naïve B cells expressing the latency III programme in the germinal centres (Babcock *et al.*, 2000; Joseph *et al.*, 2000).

Exit from the germinal centres induces a latency programme in which few or no latent genes are expressed (**Table 1.2**). Memory B cells therefore evade immune recognition and elimination by EBV-specific T cells and provide a life-long reservoir of EBV-infected cells. The EBV life cycle is completed by memory B cells reactivating and infecting new hosts. The details of this process are unclear, however it is possible that antigen-induced activation of B cells recruits memory B cells into the germinal centres of the tonsil and drives differentiation into plasma cells, a process which has been linked to activation of the viral lytic genes (Crawford and Ando, 1986). EBV particles are then produced and can be either shed into the saliva or used to replenish the EBV reservoir by infecting co-resident naïve B cells.

1.2 Immune responses to EBV and evasion strategies employed by the virus

1.2.1 General immune responses to viruses

The immune response to a pathogen involves both innate and adaptive immunity. The innate immune response represents the initial line of host defences and consists of anatomical barriers such as the skin and mucous membranes, the complement cascade, phagocytic cells such as macrophages and neutrophils, natural enzymes such as lysozyme, type I IFNs and NK cells. Most components of the innate immune response are present prior to exposure with the antigen and are not specific to a particular pathogen. Re-exposure with the same pathogen instigates an identical cascade of disease-resistant mechanisms which is uniform across all members of the same species. Upon contact with an antigen, macrophages secrete a series of factors that promote the inflammatory response and the elimination of pathogens. These include the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), IFN- α and tumour necrosis factor α (TNF- α) as well as complement proteins and hydrolytic enzymes.

The innate response to a viral infection is primarily through the induction of type I IFNs (IFN- α and IFN- β) and the activation of NK cells. Type I IFNs are secreted by infected cells and dendritic cells (via their pattern-recognition receptors, PRRs) in response to double-stranded RNA produced during the viral life cycle. IFN- α and IFN- β mediate an anti-viral response by binding to the IFN α/β receptor and activating the JAK-STAT pathway. This in turn up-regulates transcription of several host genes, including the enzyme 2'-3' oligo-adenylate synthetase [2-5(A) synthetase] which activates a ribonuclease that degrades viral RNA. Other genes activated by

IFN- α and IFN- β include dsRNA-dependent protein kinase (PKR) which prevents further viral replication by inactivating protein synthesis. Type I IFNs can also induce the adaptive immune response to a viral infection by increasing expression of costimulatory molecules such as CD80, CD86 and CD40 on the surface of dendritic cells. This in turn facilitates antigen presentation and activation of antigen-specific T cell responses. IFN- α and IFN- β in conjunction with IL-12 also bind to and activate NK cells inducing lytic activity and enhancing their capacity as cytotoxic cells.

The adaptive immune response represents an evolutionary high level of defence which is able to provide specificity, diversity, memory and recognition of self/non self. In contrast to the innate immune response, the adaptive immune response only starts after exposure to an antigen and is able to change its response following re-exposure with the same antigen. The main effectors of this response are antigen-induced T and B cells which are reliant on professional APC for activation.

Activation of naïve T cells requires two stimulatory signals. The first involves binding of the T cell receptor (TCR) to antigen that has been processed and presented on the surface of a MHC molecule as a peptide. The second signal is mediated by binding of costimulatory molecules found on the surface of all APC such as the glycoprotein B7-1 to CD28 on the surface of T cells. The presence of both signals initiates T cell activation, secretion of IL-2 and expression of the IL-2 receptor. Without a costimulatory signal, naïve T cells are unable to be fully activated and either become anergic or die by apoptosis.

Antigens are processed and presented by the MHC molecules on the surface of APCs according to the route by which they enter the cell (Morrison *et al.*, 1986). Endogenous antigens are produced from within the host cell and include viral proteins

from virally infected cells or unique tumour proteins (**Figure 1.4**). This form of antigen is degraded by the proteasome into peptides 8-10 amino acids long which are then transported into the endoplasmic reticulum (ER) by the protein transporters TAP1 and TAP2 (Powis *et al.*, 1991). Inside the ER MHC class I molecules are synthesised and stabilised by binding of β_2 microglobulin, which in turn facilitates the binding of the antigen peptide to the MHC class I peptide cleft and exit from the ER. Peptide:MHC class I complexes are then transported to the cell surface via the Golgi apparatus. The MHC-peptide complex is recognised by T cells expressing the CD8 molecule.

MHC class II molecules are also synthesised in the ER where they associate with an invariant chain protein (**Figure 1.4**). MHC class II molecules are then transported through the endocytic compartments where the invariant chain is degraded leaving a shorter fragment known as CLIP (class II-associated invariant chain peptide) within the peptide cleft. These mechanisms inhibit the possibly undesirable binding of endogenous peptide fragments.

Exogenous antigen, produced outside the host cell enters by phagocytosis and enters the endocytic compartments. Here the antigen is degraded into peptide fragments. Expression of a non-classical MHC class II molecule, HLA-DM mediates the exchange of CLIP with the peptide fragment into the binding cleft of the MHC class II molecule (Denzin and Cresswell, 1995; Sloan *et al.*, 1995). The peptide:MHC class II complex is then exported to the surface of the APC where it is recognised by T cells expressing the CD4 molecule.

Figure 1.4: Antigen presentation via the endogenous and exogenous pathways

Endogenous pathway: Endogenous antigen (1) is processed by the proteasome and degraded into peptide fragments. The peptides are then transported into the ER via TAP1 and TAP2 molecules (2). The peptide binds to the cleft present on the surface of a MHC class I molecule bound to B2 microglobulin and exits the ER into the Golgi apparatus (3). The peptide:MHC class I molecule is then transported to the surface of the APC (4).

Exogenous pathway: MHC class II molecules are synthesised within the ER and bound to an invariant chain protein (1) which inhibit binding of the peptides. The MHC class II + invariant chain are then transported to the Golgi apparatus (2) and into the endocytic compartments. Here the invariant chain dissociates (3) leaving a short fragment called CLIP. HLA-DM, a non-classical MHC class II molecule expressed in the endocytic compartments mediates the exchange of CLIP for peptide fragments (4) degraded from exogenous antigen (5). The peptides bind to the open cleft of the MHC class II molecule and are transported to the surface of the APC (6).

Figure adapted from (Goldsby *et al.*, 2003)

Evidence, however, has suggested that this model of antigen presentation is oversimplistic and that professional APC are also able to present exogenous antigen via MHC class I molecules in a process referred to as cross-presentation (Norbury and Sigal, 2003). Recent mouse studies have also suggested that this is the main mechanism by which primary viral CD8⁺ T cell responses are generated (Mueller *et al.*, 2002; Gold *et al.*, 2002).

The effector functions of the adaptive immune response can be tailored according to the invading pathogen, the microenvironment created by the infection and the means of antigen presentation. Activated CD4⁺ T-helper (T_H) cells are involved in this process by modulating their cytokine profile in response to the antigen. Two polarised responses have been described (T_H1 and T_H2 responses), although it is thought that most pathogens require a mixture of both for effective eradication.

The T_H1 response describes the secretion of IFN- γ , TNF- β and IL-2 by activated T_H cells in response to a pathogen such as an intracellular virus that requires eradication by cell mediated cytotoxicity. These cytokines activate CD8⁺ T cells and drive the proliferation of cytotoxic T lymphocytes (CTLs). CTLs kill virally infected cells by the release of cytotoxic granules containing perforin and granzymes or via the Fas-Fas ligand pathway resulting in apoptosis. The T_H1 cytokine profile also activates macrophages and induces antibody-class switching to IgG antibodies that support phagocytosis and complement fixation.

In contrast, the T_H2 response describes the secretion of IL-4, IL-10 and IL-13 from activated T_H cells in response to a pathogen such as a soluble bacterial toxin that requires neutralisation by antibodies. These cytokines provide B cell help by promoting B cell differentiation and secretion of antibodies.

B cell development and activation has been described previously (**Figure 1.3**). The net result of this process is the production of antibodies specific for an antigen. Antibodies bind to antigen through their variable regions, however, the effector functions are mediated through the constant region domains. Opsonisation, complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC) are all mediated by the antibody:antigen complex binding to the Fc or C3b receptors on phagocytes, macrophages or NK cells. Neutralisation of viruses is mediated by antibodies binding to viral epitopes that facilitate either viral attachment to the receptors on the target cell used for cellular entry or fusion of the viral envelope with the plasma membrane. If the latter occurs, the antibody is able to mediate complement driven lysis of the enveloped viral particle. Antibodies or complement may also agglutinate viral particles resulting in opsonisation and phagocytosis.

1.2.2 Innate response to EBV

Recent studies from patients with the X-linked lymphoproliferative (XLP) disease have demonstrated the role of NK cells in controlling primary EBV infection. Young males with the genetic disposition to XLP are clinically well until primary EBV infection when 50% develop fulminant IM, 20% develop B cell lymphomas and 30% develop dys-gammaglobulinaemia (Howie *et al.*, 2000; Hamilton *et al.*, 1980). All three phenotypes have been shown to be secondary to a deregulated EBV-specific T cell response and uncontrolled B cell proliferation. The genetic defect in XLP is an absence of the signalling lymphocytic activation molecule (SLAM) associated protein (SAP) (Coffey *et al.*, 1998; Sayos *et al.*, 1998; Nichols *et al.*, 1998), a protein which is thought to control NK activation by interacting with the NK costimulatory receptor

2B4/CD244. This receptor is constitutively expressed on NK cells (Nakajima *et al.*, 2000) and has been shown *in vitro* to bind to CD48 (Brown *et al.*, 1998), a molecule highly expressed on B cells shortly after infection with EBV (Thorley-Lawson *et al.*, 1993). It has therefore been postulated that NK cells recognise EBV through the CD244/CD48 interaction and that abnormal signalling in XLP due to the absence of SAP leads to failure of NK cell control of primary EBV infection.

1.2.3 B cell responses to EBV

EBV stimulates a strong antibody response during primary infection. Early in the diagnosis of IM, IgM and IgG responses to the nucleocapsid and envelope proteins are evident. This is typically followed by IgG responses to the lytic proteins as well as EBNA2 and EBNA1 (Nikoskelainen and Hanninen, 1975). Neutralising antibodies to gp350 (the point of entry for EBV into the B cells) arise late in the course of IM, and therefore do not play an important role in the control of the virus.

1.2.4 T cell responses to EBV

The cell-mediated response to EBV has been well documented and shown to play a critical role in the control of primary and persistent EBV infection and prevention of uncontrolled EBV-driven B cell proliferation. CD8⁺ T cell and CD4⁺ T cell responses are considered below.

1.2.4.1 CD8⁺ T cell responses

Primary EBV infection is associated with large clonal expansions of CD8⁺ T cells (Callan *et al.*, 1996; Maini *et al.*, 2000) that are predominately directed to the lytic EBV antigens (Callan *et al.*, 1998b; Hislop *et al.*, 2002). Studies with fluorochrome

labelled recombinant HLA class I:peptide complexes, known as tetramers, to detect EBV specific T cell in patients with IM showed that up to 12% of circulating CD8⁺ T cells were specific for an HLA-A2 restricted epitope (GLCTLVAML) from the lytic antigen BMLF1, and up to 45% were specific for an HLA-B8 restricted epitope (RAKFKQLL) from the intermediate early lytic antigen BZLF1. In contrast, the magnitude of the CD8⁺ T cell response to latent EBV antigens is considerably smaller and typically comprises of 2.5% of the peripheral CD8⁺ T cell population (Callan *et al.*, 1998b; Callan *et al.*, 1998b; Hislop *et al.*, 2002). Explanations for why primary CD8⁺ T cell responses should be directed towards lytic EBV antigens during primary infection are unclear, however a recent study suggested that the efficiency of antigen presentation may be responsible (Pudney *et al.*, 2005).

Functional studies on the clonally expanded CD8⁺ T cells have indicated their protective role in primary EBV infection. EBV-specific CD8⁺ T cells express a variety of activation markers (Callan *et al.*, 1998b; Hislop *et al.*, 2002) and have been shown to demonstrate efficient cytolytic activity *in vitro* (Callan *et al.*, 2000; Steven *et al.*, 1996). In addition, on stimulation with HLA-restricted peptides, a proportion of EBV-specific CD8⁺ T cells will produce IFN- γ (Hoshino *et al.*, 1999).

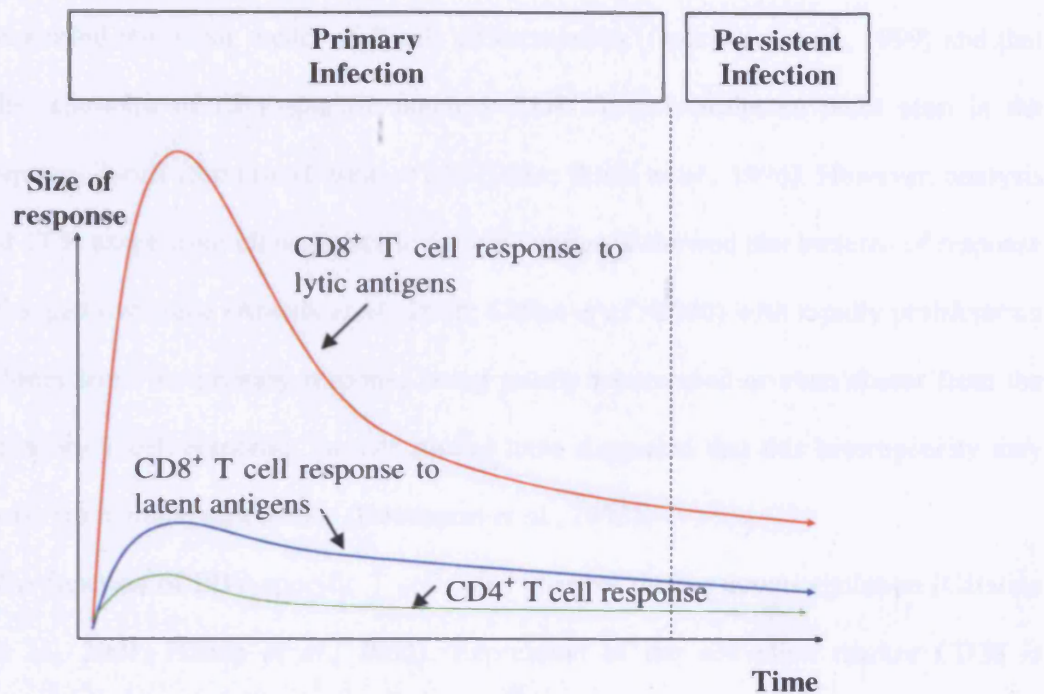


Figure 1.5: The dynamics of T cell responses to EBV

Following the large primary burst of responses towards lytic antigens in primary infection and smaller response to latent antigens, T cell responses are down regulated (**Figure 1.5**). Evidence has shown that EBV-specific CD8⁺ T cells die *in vitro* by apoptosis pathways independent of Fas-ligand or TRAIL, and that they express low levels of Bcl-2, an intracellular molecule that protects against apoptosis mediated by cytochrome c (Callan *et al.*, 2000). This suggests therefore that EBV-specific CD8⁺ T cells are programmed for cell death and will die after clonal expansion in the absence of survival signals.

The mechanisms involved in the selection of which T cells from the primary clonal response survive and are represented in the memory T cell population, and which T cell clones die by apoptosis are poorly understood. Initial evidence analysing the TCR

usage of latent antigen CD8⁺ T cells in primary and persistent EBV infection supported the linear model of T cell differentiation (Opferman *et al.*, 1999) and that the repertoire of EBV-specific memory CD8⁺ T cells reflected those seen in the primary T cell response (Callan *et al.*, 1998a; Silins *et al.*, 1996). However, analysis of TCR usage from clones specific for lytic antigens showed that patterns of response changed over time (Annels *et al.*, 2000; Callan *et al.*, 2000) with rapidly proliferating clones from the primary response being poorly represented or even absent from the memory T cell response. Further studies have suggested that this heterogeneity may arise from clonal senescence (Davenport *et al.*, 2002).

The function of EBV-specific T cells also changes during down-regulation (Catalina *et al.*, 2001; Hislop *et al.*, 2002). Expression of the activation marker CD38 is typically lost, while expression of CD62L (L-selectin) and the chemokine receptor, CCR7 increase, changes which reflect maturation of the immune response.

Persistent EBV infection evokes a similar pattern of T cell responses to that seen in primary EBV infection with lytic antigen-specific T cells dominating over latent antigen responses. Together both lytic and latent CD8⁺ T cells responses account for 1-3% of the circulating CD8⁺ pool in healthy EBV sero-positive individuals (Tan *et al.*, 1999); a relatively high frequency which reflects the response to intermittent viral reactivation. Studies have shown however that not all of these cells appear to be functionally equivalent, as demonstrated by the frequency of IFN- γ producing EBV peptide-specific CD8⁺ T cells representing 20-40% of the epitope-specific CD8⁺ T cells measured by HLA tetramer staining for the same epitope (Tan *et al.*, 1999). A second study also illustrated functional heterogeneity in persistent infection by showing that IFN- γ producing lytic peptide-specific CD8⁺ T cells do not always

express the activation marker CD28 or the CD45RO isotype consistent with the classical model of central memory differentiation (Hislop *et al.*, 2001). Recent evidence however suggests that a proportion of memory EBV-specific CD8⁺ T cells that produce IFN- γ in response to peptide stimulation and are cytolytic *in vitro* also re-express the CD45RA isoform (Dunne *et al.*, 2002). These cells are resistant to apoptosis due to increased expression of bcl-2 and therefore may represent a subpopulation of memory T cells that are crucial in preserving the long-term immunity of EBV.

1.2.4.2 CD4⁺ T cell responses

Analysis of the CD4⁺ T cell response to EBV has lagged behind that of the CD8⁺ response, primarily due to restricted knowledge of epitopes binding to the MHC class II molecule, and the limited availability of class II HLA tetramers.

Despite this, several studies have used PBMCs stimulated with lysates from EBV-transformed lymphoblastoid cell lines (LCLs) or fusion proteins to stimulate a CD4⁺ response from patients with primary infection. The first study showed that up to 5.2% (mean of 1.4%) of circulating CD4⁺ T cells responded to EBV antigen by producing IFN- γ (Amyes *et al.*, 2003). The second study, however, demonstrated lower frequencies of IFN- γ producing CD4⁺ T cells after stimulation with fusion proteins containing both lytic and latent EBV proteins (Precopio *et al.*, 2003). CD4⁺ T cell responses were directed to the BZLF1 and BMLF1 lytic proteins and EBNA1 latent proteins, with little response to EBNA 3A. In both studies, responses had fallen to undetectable levels by 1 year, suggesting that CD4⁺ T response to EBV in primary

infection is characterised by a small clonal burst followed by a period of downregulation (**Figure 1.5**).

Phenotypic analyses of CD4⁺ T cells from patients with persistent infection have shown that functional heterogeneity also exists in this population as well as the CD8⁺ memory T cells (Amyes *et al.*, 2005; Harari *et al.*, 2005). Results showed that EBV-specific CD4⁺ T cells could be compartmentalised according to their expression of CCR7, CD27 and CD28 as well as their ability to produce the cytolytic mediators, perforin and granzyme and the cytokines IFN- γ , TNF- α and IL-2. These studies therefore provide insights into CD4⁺ T cell differentiation following exposure to antigen.

1.2.5 EBV immune evasion strategies

1.2.5.1 Latent gene down regulation

The main immune evasion mechanism that EBV employs during latent infection is down regulation of latent antigen expression. Infected memory B cells express no or little EBV antigens (Latency programme 0, **Table 1.2**) (Babcock *et al.*, 1998; Hochberg and Thorley-Lawson, 2005b) therefore limiting the number of targets that EBV-specific CD8⁺ T cells are able to recognise. In contrast, EBV infected B cells that enter the lytic cycle for viral replication, express all the latent antigens (latency programme III). These antigens prompt vigorous CD8⁺ T cell responses and cell mediated killing (Rickinson and Moss, 1997). This therefore ensures that EBV-driven proliferation of B cells is controlled and that persistent EBV infection remains a benign event for the host.

1.2.5.2 Inhibition of antigen processing by Gly-Ala repeat

As previously discussed the Gly-Ala repeat domain found in EBNA1 is able to inhibit antigen presentation to CD8⁺ T cells by inhibiting proteasomal processing (Levitskaya *et al.*, 1995). Therefore EBV-associated malignancies such as Burkitt's lymphoma that express EBNA1 alone and proliferating memory B cells in healthy individuals avoid recognition by EBV-specific CD8⁺ T cells and are rendered immunologically invisible. Recent studies however, have challenged this, showing that EBNA1-derived epitopes derived from defective proteins that are prematurely truncated during translation or incorrectly folded after translation (defective ribosomal products, DRiPs) can be presented successfully to CD8⁺ T cells by MHC class I molecules (Voo *et al.*, 2004; Tellam *et al.*, 2004; Lee *et al.*, 2004).

1.2.5.3 Inhibition of Protein Kinase (PKR)

EBERs are the most abundant of all the latent EBV gene products and found in all EBV-associated malignancies (**Table 1.2**). EBV utilises this protein in immune avoidance by inhibiting PKR (Nanbo *et al.*, 2002). This enzyme is activated by type I IFNs binding to the IFN α/β receptor and in conjunction with 2-5(A) synthetase prevents protein synthesis, thereby effectively blocking viral replication. EBERs however, promote EBV persistence by directly blocking PKR activity.

1.2.5.4 Suppression of innate and adaptive immunity

Several of the EBV gene products share both sequence and functional homology with host proteins. EBV can therefore mimic the activity of these proteins and modulate the immune response to the virus.

BARF1, a gene expressed early in EBV replication neutralises colony-stimulating factor-1 (CSF-1). This cytokine is responsible for both inducing monocyte proliferation as well as up regulating monocyte secretion of IFN- α (Cohen and Lekstrom, 1999). Therefore EBV can interfere with the anti-viral effects of innate immunity and delay immune responses that are dependent on monocyte proliferation and differentiation.

The EBV induced gene 3 (EBI3) is expressed at high levels by EBV-transformed B cells and has homology with the p40 subunit of IL-12 (Devergne *et al.*, 1997). IL-12 is essential for NK cell activation and when released from professional APCs polarises specific T cell responses towards a T_H1 phenotype. It has therefore been proposed that EBI3 acts as an immunomodulator as well as an inhibitor of NK cell activation (Devergne *et al.*, 2001).

BCRF1 is expressed early in EBV replication and shares 85% homology with IL-10 (Hsu *et al.*, 1990). This gene is therefore able to suppress T cell responses directly by reducing IL-2 and IFN- γ production and indirectly by regulating MHC class I and II expression and suppressing professional APC migration to regional LNs. EBERs present in both lytic and latent EBV infection have also been shown to exert the same effects by up regulating IL-10 production (Nanbo *et al.*, 2002).

BHRF1 shares 25% sequence homology with the human bcl-2 proto-oncogene (Henderson *et al.*, 1993) and has been shown to inhibit apoptosis by interfering with granzyme mediated cell killing by CTLs (Davis *et al.*, 2000).

1.3 EBV associated malignancies: The role of EBV in pathogenesis

EBV is associated with both lymphoid and epithelial malignancies. However, despite EBV infecting 90% of the world-wide population, it is only responsible for an estimated 1-2% of malignancies. EBV-related malignancy is also often confined to geographical areas. This therefore suggests that although EBV is involved in tumour development, additional cofactors are also required. The following sections outline the proposed pathogenesis of both lymphoid and epithelial EBV-associated malignancies by describing their cell phenotype and how this relates to the role of EBV in persistent infection of healthy individuals detailed previously.

1.3.1 EBV-associated lymphoid malignancies

EBV is associated with B-cell as well as T and NK cell lymphomas. Each express different patterns of latent genes (**Table 1.2**) and originate from different cell phenotypes within the differentiation pathways. Individual sets of cofactors also appear to be vital for the pathogenesis of each type of lymphoma.

1.3.1.1 Burkitt's Lymphoma

Burkitt's lymphoma (BL) exists in two forms. The endemic form was originally described as a tumour of the jaw affecting children in equatorial Africa and New Guinea where malaria is holoendemic (BURKITT, 1958), and is associated with EBV in all cases. The endemic form of BL has a high incidence and accounts for 16% of all childhood cancers in these geographical areas (Lavu *et al.*, 2005). The sporadic form however, has a much lower incidence and by definition occurs outside equatorial Africa. In this form EBV is only found in 15% of cases (Rickinson and Kieff, 2001).

BL typically develops in extra-nodal sites such as the jaw, ovary, breast, liver and kidneys. However, histologically BL cells resemble germinal centre B cells, sharing the same phenotypic markers (CD10, CD77, BCL6) and showing evidence of ongoing somatic hypermutation.

Irrespective of EBV association or geographical location, BL cells share similar genetic abnormalities and it is thought that this is a crucial cofactor in the development of BL. The hallmark of BL is one of three chromosomal translocations which places the *c-myc* cellular oncogene on chromosome 8 under the control of the Ig heavy chain or light chain genes resulting in its constitutive expression and uncontrolled proliferation (Zech *et al.*, 1976). BL is also associated with mutations in tumour suppressor genes such as p53 (Bhatia *et al.*, 1992; Gaidano *et al.*, 1991) and retinoblastoma-like 2 (RB2) (Cinti *et al.*, 2000).

The role of EBV in the pathogenesis of BL is less certain. EBV-positive BL cells only express EBNA1, a latent gene that is primarily involved in the maintenance of the viral genome (Yates *et al.*, 1985) and with questionable oncogenic potential. It is therefore possible that BL arises from EBV-infected germinal centre B cells that are destined to becoming a memory B cell but that *c-myc* expression occurring as an error of somatic hypermutation prevents differentiation and promotes proliferation of B cells expressing the restricted latency type I pattern of EBV antigens. This model is illustrated in **Figure 1.6** and suggests that EBV has no role in the oncogenesis of BL and is present solely by chance. Alternatively it is possible that the non-coding EBER RNAs contribute to BL pathogenesis by inducing the anti-apoptosis gene *bcl-2* and promoting cell survival. Recent evidence has shown that EBER RNA expression is up regulated by *c-myc* (Niller *et al.*, 2003) and therefore sustained EBER expression by

BL cells may counteract the pro-apoptotic function of *c-myc* and allow *c-myc*-driven proliferation.

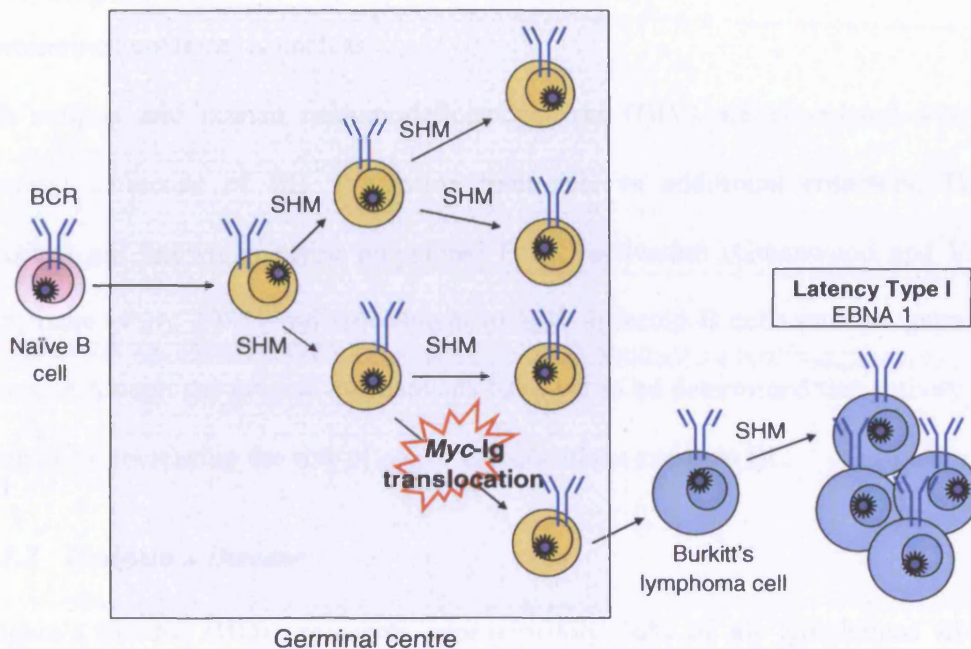


Figure 1.6: Bystander pathogenesis model of Burkitt's lymphoma

EBV infected naïve B cells proliferate within the germinal centre where they undergo somatic hypermutation (SHM). During this process B cells that were destined to become memory B cells are vulnerable to translocation of the proto-oncogene, *Myc* into the immunoglobulin loci (*Myc*-Ig translocation), resulting in uncontrolled proliferation.

★ EBV viral particle

Another option is that EBV initiates oncogenesis by transforming germinal centre B cells that are then targets of *c-myc* translocations. These cells would therefore initially express all latent genes, however subsequent selection forces down regulation of EBV genes to latency type I expression. Evidence from this model comes from a recent study showing deletion of the growth promoting EBNA2 gene in several cases of BL (Kelly *et al.*, 2002). Whether this selection pressure results from the incompatibility

that exists *in vitro* between EBV latency type III-driven and *c-myc*-driven growth programmes in B cells (Pajic *et al.*, 2001), or a need to down regulate immunogenic EBV proteins in immuno-competent hosts, or the expression of another as yet undetermined cofactor, is unclear.

Both malaria and human immunodeficiency virus (HIV) are associated with an increased incidence of BL, suggesting their role as additional cofactors. These infections are known to cause polyclonal B cell activation (Greenwood and Vick, 1975; Lane *et al.*, 1983) and recruitment of EBV-infected B cells into the germinal centres. Although the precise mechanisms have yet to be determined this activity has potential for increasing the risk of *c-myc* translocations and thus BL.

1.3.1.2 Hodgkin's Disease

Hodgkin's disease (HD) represents approximately 20% of all lymphomas in the western world and has a rising incidence. The role of EBV in the pathogenesis of HD is unclear, however, EBV clonal DNA is found in the R-S cells of approximately 40-50% of HD tumours.

The characteristic histopathological features of HD are small numbers of the malignant multi-nucleated Reed-Sternberg (R-S) cells and Hodgkin cells surrounded by large populations of mononuclear cells. It is this infiltrate that is used to classify HD into two histological groups: Classical HD (lymphocyte-depleted HD; nodular sclerosing HD; mixed cellularity HD and lymphocyte-rich HD) and Non-classical HD or nodular lymphocyte predominant HD (Harris *et al.*, 1994).

The cellular origins of R-S cells in classical HD have long been debated, however, recent molecular analyses have identified them as germinal centre B cells that contain

functional immunoglobulin rearrangements but with nonsense mutations or deletions resulting in defective Ig transcription (Kuppers, 2002; Kanzler *et al.*, 1996; Marafioti *et al.*, 2000). These mutations would ordinarily ensure elimination by apoptosis in the germinal centres (**Figure 1.7**) suggesting that HD results from these atypical cells being rescued by a transforming agent. EBV-positive R-S cells express LMP1 and LMP2 in addition to EBNA1 (Latency pattern II). Both LMP genes are able to rescue B cells from apoptosis by activating the CD40 and BCR signalling pathways as discussed previously and therefore indicate a probable role for EBV in the pathogenesis of EBV-positive tumours. Whether EBV is involved in the established R-S cell clone in HD is unclear, particularly as there are no latency type II expressing EBV-positive HD cell lines available for study. R-S cells have lost several downstream components of the BCR signalling pathway (Schwering *et al.*, 2003) and therefore LMP2 is unlikely to function in the established tumour, at least through the recognised pathways. In contrast, LMP1 continues to exert its effects through the activation of NF- κ B. Similar deregulation of NF- κ B function is also seen in EBV-negative R-S cells, with inhibition of the main NF- κ B regulator (Kuppers, 2002) indicating the importance of this pathway in the pathogenesis in both EBV-positive and negative HD.



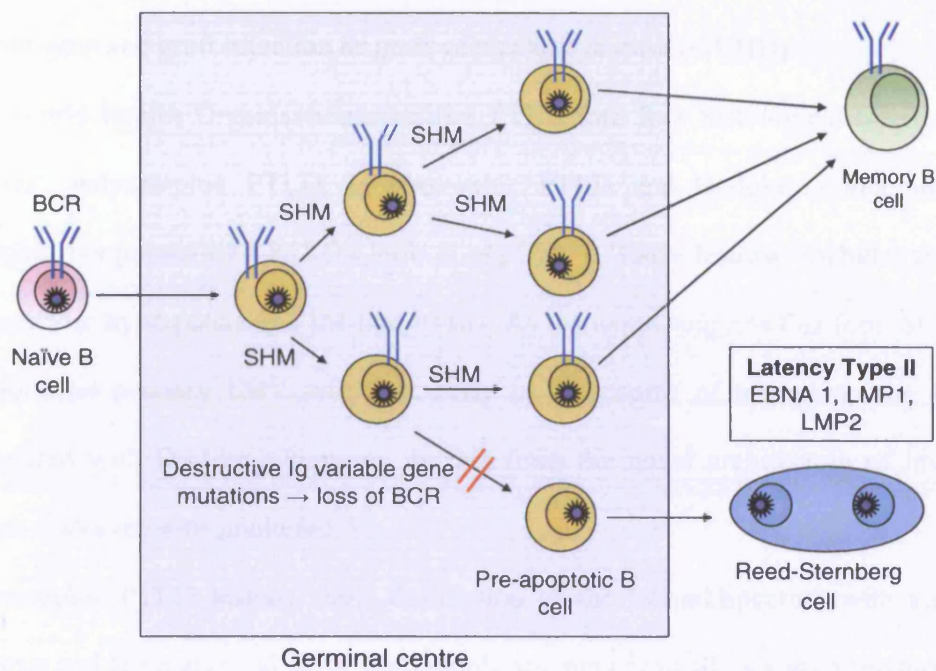


Figure 1.7: Pathogenesis of EBV-positive HD

The malignant R-S cells of HD originate from germinal centre B cells with destructive Ig variable gene mutations. These cells normally are eliminated by apoptosis but are thought to be rescued by survival signals from the EBV latent genes LMP1 and LMP2.



= EBV viral particle SHM= Somatic hypermutation

1.3.1.3 Post transplant lymphoproliferative disease

Post transplant lymphoproliferative disease (PTLD) is a heterogeneous group of B cell lymphoproliferative disorders together with a small number of T and NK lymphomas which occur in immunocompromised patients whose defective T cell immunity is unable to control EBV-driven B cell proliferation. Commonly these lymphomas occur after solid organ and haematopoietic stem cell transplantation

where immunosuppressive drugs are required to suppress T cell immunity to alloantigens and graft rejection or graft versus host disease (GVHD).

The World Health Organisation classifies PTLD into four histological types: ‘early lesions’, polymorphic PTLD, monomorphic PTLD and Hodgkin lymphoma and Hodgkin lymphoma-like PTLD (Jaffe *et al.*, 2001). ‘Early lesions’ includes reactive plasmacytic hyperplasia and IM-like PTLD. As the name suggests this form of PTLD occurs after primary EBV infection, early in the course of transplantation and is associated with IM-like symptoms. In this form the nodal architecture of involved lymph nodes remains unaltered.

Polymorphic PTLD lesions show destruction of the LN architecture with areas of necrosis and large atypical cells. Immunophenotyping typically shows a mixture of B and T cells. Monomorphic PTLD tumours usually resemble diffuse large B cell non-Hodgkin’s lymphomas, however lesions resembling Burkitt’s or Burkitt’s-like lymphoma, plasma cell myeloma, plasmacytoma and T-cell lymphomas are also seen. EBV is associated with PTLD in 71% of cases (P.Amlot , personal communication), however its precise role in pathogenesis remains undefined. The majority of lesions are monoclonal (Knowles *et al.*, 1995; Chadburn *et al.*, 1998) and although were previously thought to express an unrestricted pattern of EBV latent gene expression similar to LCLs (Thomas *et al.*, 1990; Young *et al.*, 1989), research has now shown that PTLD lesions express different patterns of EBV latency (**Table 1.2**), with variable expression of EBNA 2 and LMP common (Cen *et al.*, 1993; Timms *et al.*, 2003). Ig variable (IgV) gene analysis has shown that EBV-positive PTLD lesions can arise from naïve cells, memory cells and atypical germinal centre B cells that have destructive Ig mutations that are normally inconsistent with cell survival (Timms *et*

al., 2003; Capello *et al.*, 2005; Brauning *et al.*, 2003). This demonstrates that EBV-positive PTLD can arise from many points along the B cell development pathway both within the germinal centres and post-germinal centre compartments (**Figure 1.8**) and illustrates similarities with EBV-positive HD (Timms *et al.*, 2003), suggesting a common initiation event may be performed by EBV. One hypothesis is that EBV creates a pool of non-malignant atypical B cells either by rescuing cells from within the germinal centre or activating SHM in post-germinal B cells. Malignant transformation may then occur as a result of additional genetic mutations. In support of this are the findings that EBV-positive PTLD is commonly associated with BCL-6 (Cesarman *et al.*, 1998) mutations as well as mismatch repair deficiency (Duval *et al.*, 2004). Mutations of other oncogenes such as c-myc, N-ras and p53 occur sporadically however, and show no consistent association.

Others have suggested that a cytokine imbalance from persistently activated CD4⁺ T cells may provide the cofactor required for the malignant transformation of EBV-infected B cells. Activated CD4⁺ T cells stimulated by alloantigens in the context of transplantation produce a spectrum of cytokines which may promote atypical B cell survival and proliferation. This suggestion is supported by the infiltration of CD4⁺ T cells in the majority of PTLD lesions (Perera *et al.*, 1998) as well as experiments on severe combined immunodeficient (SCID) mice showing that the development of EBV-positive B-cell lymphoproliferative disease-like lesions after injection with mononuclear cells from EBV sero-positive healthy individuals is dependent on the presence of donor CD4⁺ T cells (Johannessen *et al.*, 2000).

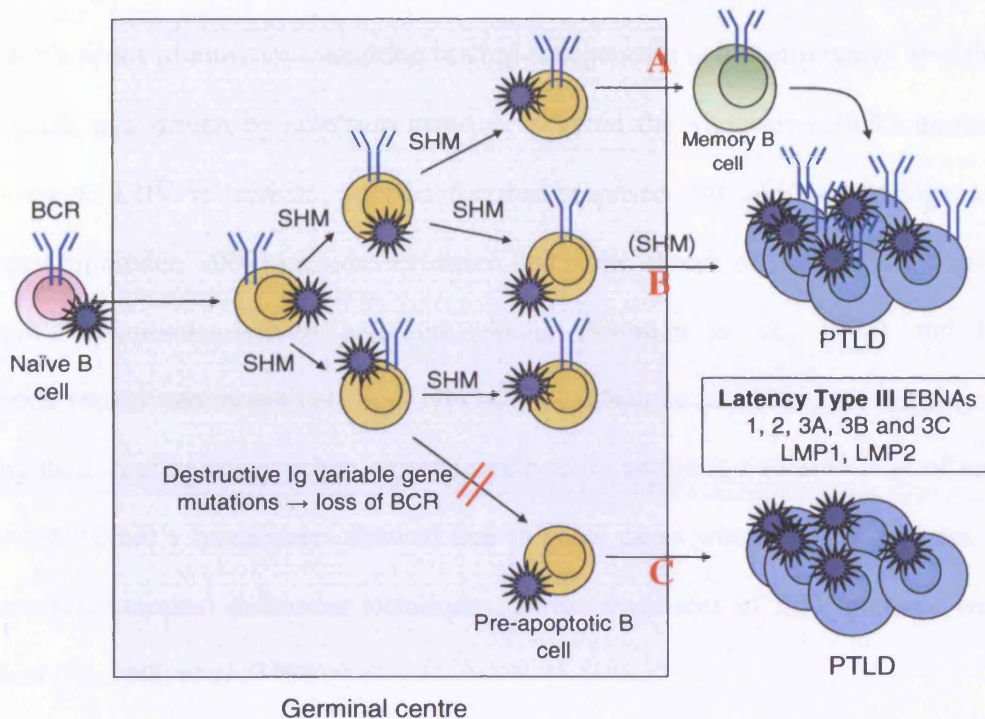


Figure 1.8: Pathogenesis of EBV-positive PTLD

PTLD lesions are heterogeneous in terms of cellular origin. Lesions may arise from memory B cells (A), naïve B cells (B) or atypical B cells that are rescued from apoptosis within the germinal centre (C).

☼ = EBV viral particle

EBV-negative PTLD accounts for 29% of all PTLD lesions (P.Amlot, personal communication). These tumours usually occur late after transplantation and are often more aggressive resulting in a poor prognosis compared to EBV-positive PTLD (Leblond *et al.*, 1998). The pathogenesis for EBV-negative PTLDs has yet to be fully elucidated. Some studies have suggested that they represent sporadic lymphomas that have occurred in immunosuppressed patients and should not be considered under the PTLD classification umbrella (Dotti *et al.*, 2000; Sivaraman and Lye, 2001). Other studies however, have suggested that EBV was initially involved in the oncogenesis

of EBV-negative PTLT tumours but loss of the EBV antigen subsequently occurred through a series of mutations resulting in viral-independent cell proliferation. Whether this event was driven by selection pressure to avoid the vigorous cellular immune response to EBV is unclear. Support for this proposed 'hit and run' oncogenesis theory (Ambinder, 2000) includes evidence that some clones of the EBV-associated Burkitt's lymphoma-derived cell line, Akata (Shimizu *et al.*, 1994) and the nasopharyngeal carcinoma cell line, NPC-C666, (Chen *et al.*, 1999) are capable of losing their viral episomes when grown in culture. In addition, a recent series of non-endemic Burkitt's lymphomas showed that in some cases where EBV could not be detected by standard molecular techniques, partial fragments of EBV genome were evident (Razzouk *et al.*, 1996).

1.3.1.4 T-cell and NK-cell lymphomas

EBV preferentially infects and transforms B cells *in vitro* and therefore the association of the virus with specific forms of T-cell and NK-cell lymphomas was a surprise finding (Harabuchi *et al.*, 1990; Jones *et al.*, 1988). Peripheral T cell lymphomas are rare and are most common in South-East Asia. The lymphomas show CD4⁺ or CD8⁺ T cell phenotypes and typically express latency type I or II programmes. This type of T cell lymphoma can either arise after acute primary EBV infection when it manifests as a haemophagocytic disorder or as a sequelae of chronic active EBV (Kimura *et al.*, 2003; Kanegane *et al.*, 2002), a rare life threatening disorder characterised by recurrent or persistent-IM like symptoms (fever, fatigue lymphadenopathy and hepatosplenomegaly) in combination with an elevated EBV viral load and an abnormal EBV antibody profile. Mortality from chronic active EBV

is high (40%) with the majority of patients dying from hepatic failure, haemophagocytic disorders or lymphomas (Kimura *et al.*, 2001).

EBV is also associated with an extra-nodal lymphoma which presents as a lethal mid-line granuloma in the nasal cavity (Kanavaros *et al.*, 1993). This lymphoma is most common in south-east Asia and can arise from both T and NK-cells. Latent EBV antigen expression typically follows the type II programme.

The role of EBV in the pathogenesis of these lymphomas has yet to be fully determined, however EBV infected T cells *in vitro* express the same CD21 receptor required for EBV entry into B cells (Fingerroth *et al.*, 1988) suggesting that T cell lymphomas may arise from a pre-malignant pool of T cells *in vivo*.

1.3.2 EBV associated epithelial malignancies

1.3.2.1 EBV infection of epithelial cells

EBV is associated with epithelial malignancies (NPC and gastric carcinoma) however, the mechanism by which EBV infects epithelial cells remains poorly understood. Squamous epithelial cells lack the CD21 receptor used by EBV to access B cells and are resistant to EBV infection *in vitro* (Sixbey *et al.*, 1984; Imai *et al.*, 1998). This finding led to the suggestion that EBV must undergo a preliminary round of replication in the B cells of the oropharynx before the virus can access the epithelial cells. However, recent evidence has shown not all the EBV viral particles are internalised on binding to the surface of B cells and that EBV infection can be transferred directly to the epithelial cells by B cells through gp350/CD21 complexes (Shannon-Lowe *et al.*, 2006).

1.3.2.2 *Nasopharyngeal carcinoma*

Nasopharyngeal carcinoma (NPC) arises from the squamous epithelium of the post-nasal space and is classified according to the degree of differentiation of the malignant cells. 70% are undifferentiated which are invariably associated with EBV (Vasef *et al.*, 1997). Undifferentiated NPC is rare in most regions of the world but with a particularly high incidence in certain areas of China and south-east Asia, reaching a level of 20-30 cases per 100,000 (Yu and Yuan, 2002). In addition to a preponderance for geographical regions, the aetiology of NPC has also been associated with dietary factors such as salted fish (Armstrong *et al.*, 1998; Ning *et al.*, 1990). Early stage NPC is curable in the majority of patients with a combination of radiotherapy and chemotherapy, however the prognosis of patients with locally advanced or metastatic disease remains poor (Chan *et al.*, 2002). Recent clinical studies however have shown some therapeutic benefits with adoptive CTL therapy in patients with advanced disease (Straathof *et al.*, 2005; Comoli *et al.*, 2005).

The point in time at which EBV infects epithelial cells *in vivo* remains controversial. The epithelial cells of undifferentiated NPC carry monoclonal viral genomes which suggests that EBV infection occurs before the outgrowth of the malignant clone (Raab-Traub and Flynn, 1986). However, although EBV has been identified in high grade pre-invasive lesions such as carcinoma *in situ* and severe dysplasia, it is not present in normal epithelial cells from patients at risk of developing NPC or in low-grade pre-invasive lesions (Pathmanathan *et al.*, 1995). This finding argues against the premise that EBV-positive NPC develops from a pre-existing normal reservoir of EBV infected epithelial cells. Interestingly genetic defects (deletions of the short arms of chromosomes 3 and 9) have been found in normal epithelial cells of patients at

high risk of developing NPC as well as frankly malignant epithelial cells (Lo and Huang, 2002). These findings have raised the possibility that this loss of heterozygosity on chromosomes 3p and 9p may result from environmental cofactors such as continued dietary exposure to salted fish and that this leads to the development of low-grade pre-invasive lesions. Following a series of additional genetic and epigenetic events these lesions are then predisposed to EBV infection. Subsequent latent EBV gene expression could then promote cell survival and proliferation resulting in the development of NPC. This possible sequence of events is supported by *in vitro* data which shows that the stable maintenance of EBV in epithelial cells requires an altered undifferentiated cellular environment (Knox *et al.*, 1996).

Undifferentiated NPC expresses a restricted pattern of viral latent genes according to latency programme type II. Despite normal antigen processing and efficient recognition by EBV-specific CTLs, these cells are not destroyed *in vivo*. Possible explanations for this immune evasion include an increase in EBV-encoded IL-10 leading to an increased production of IL-1 α and IL- β by both epithelial cells and surrounding CD4⁺ T cells (Bejarano and Masucci, 1998; Huang *et al.*, 1999): two cytokines that have been linked with increased cell survival. Bcl-2 over-expression is also seen in NPC and may contribute to oncogenesis by allowing the cell to bypass apoptosis (Lu *et al.*, 1993).

1.3.2.3 Gastric carcinoma

EBV is associated with approximately 10% of the common gastric adenocarcinomas and over 90% of the rarer lymphoepithelioma-like gastric carcinomas (Tokunaga *et*

al., 1993; Shibata and Weiss, 1992; Takada, 1999). The latent viral expression of EBV-positive gastric adenocarcinomas is similar to NPC, however, LMP-1 is usually absent while expression of BARF1, a homologue of human colony-stimulating factor 1 is common (Imai *et al.*, 1994). The precise role of EBV in the pathogenesis of gastric carcinoma is unknown however as with NPC, EBV has not been detected in the normal gastric mucosa of high-risk patients or pre-malignant lesions (zur Hausen *et al.*, 2004), suggesting that EBV infection occurs late in the oncogenesis of this disease and is reliant on pre-existing genetic alterations within the gastric epithelial cells.

1.4 Clinical Management of PTLT

PTLT is a life-threatening complication of both solid organ transplantation (SOT) and haematopoietic stem cell transplantation (HSCT) and is caused by an ineffective cellular immune response to EBV resulting in uncontrolled B cell proliferation. In SOT patients ineffective anti-EBV immunity is caused by immunosuppressive drugs administered after transplantation to control graft rejection. In HSCT patients however, anti-EBV immunity is suppressed by a combination of high-dose chemotherapy and/or bone marrow irradiation given as part of the conditioning regime, T cell depletion from the graft and immunosuppressive drugs: therapies used to reduce the incidence of GVHD. Due to the nature of the graft PTLT arising in SOT patients is typically of recipient origin whereas PTLT in HSCT patients is of donor origin.

1.4.1 Incidence and risk factors

The incidence of PTLT in both SOT and HSCT patients is highest in the first year after transplantation due to the intensive immunosuppression required during this time to control alloreactivity (Curtis *et al.*, 1999; Bhatia *et al.*, 1996; Ho *et al.*, 1988; Sokal *et al.*, 1997). In SOT patients the incidence increases if the recipient is sero-negative at the time of transplantation and receives a sero-positive graft; a scenario common in paediatric patients, or if prolonged intensive immunosuppression is required (Cox *et al.*, 1995; Shapiro *et al.*, 1999). The incidence also varies according to the type of graft received (**Table 1.3**). In adult recipients the incidence is highest amongst small bowel transplants (up to 20%) followed by lung transplants (4.2-10%) and heart-lung transplants (2.4-5.8% (Taylor *et al.*, 2005). The incidence of PTLT in renal transplants ranges from 1-2.3% (Libertiny *et al.*, 2001; Shapiro *et al.*, 1999). Reasons for these differences in incidence are unclear, however the intensity of the immunosuppressant regimes used as well as the amount of lymphoid tissue in the different graft types may be important factors.

In HSCT patients the incidence after allogeneic transplant is approximately 1% (Bhatia *et al.*, 1996; Curtis *et al.*, 1999). The risk factors are listed in **Table 1.3** and include pure T-cell depletion as opposed to combined T- and B-cell depletion with agents such as the monoclonal anti-CD52 antibody (CAMPATH) (Cavazzana *et al.*, 1998; Hale *et al.*, 1998).

<i>Solid organ transplant</i>	<i>Haematopoietic stem cell transplant</i>
EBV sero-negative recipient receiving a graft from an EBV sero-positive donor	T-cell depletion
Small bowel or heart-lung transplant	HLA-mismatched transplant
Intensive immunosuppressive drugs	Anti-thymocyte globulin for prophylaxis of GVHD
	Underlying immunodeficiency

Table 1.3: Risk factors for developing PTLD

1.4.2 Clinical features

PTLD presents with a diverse spectrum of clinical symptoms and signs and the index of suspicion for diagnosing this disorder should be high in all transplant patients. Commonly patients present with the non-specific symptoms such as fever, malaise, tonsillitis and weight loss, however, rarely PTLD presents as a fulminant disseminated disease resembling septic shock. The symptoms of PTLD in children, who are commonly EBV sero-negative at the time of transplant, are often similar to those of primary EBV infection (infectious mononucleosis) which usually occurs in the first weeks following transplantation, with fever, tonsillar and adenoid hypertrophy, cervical lymphadenopathy and hepatomegaly typically with raised hepatic enzymes. Biopsy and histological examination is therefore necessary to distinguish between the two disorders.

PTLD can present as solitary or multiple tumours, affecting both nodal and extra-nodal sites. Common extra-nodal sites include the kidneys, liver, lungs, bowel, mediastinum, skin, bone marrow and spleen. The central nervous system (CNS) can

also be involved. A recent series of 910 adult and paediatric PTLT patients showed that CNS involvement occurred in 15% of cases and with half of these cases presenting as an isolated tumour (Buell *et al.*, 2005). This contrasts to non-Hodgkin's lymphoma (NHL) in the immunocompetent population where the incidence of disease confined to the CNS is 0.28 per 100,000 (Cote *et al.*, 1996).

1.4.3 Diagnosis

The diagnosis of PTLT is made on histological examination of the tumour. In-situ hybridisation for EBERs is used to determine the presence of EBV in the cells (Howe and Steitz, 1986) together with immunostaining for LMPs. Characterising the cellular infiltrate of the tumour for B-cell markers such as CD19, CD20, CD21 or CD22 also helps distinguish PTLT from acute rejection, which often appear similar using conventional histological staining. Clonality of the tumour is also assessed by immunophenotyping and the expression of kappa or lambda chains on the surface of the immunoglobulin molecules on the B cells.

Following diagnosis patients are subjected to a CT of the thorax and abdomen and a bone marrow aspiration to ascertain other sites of disease. Once all sites of disease have been localised, the PTLT is staged according to the Ann Arbor classification system (Carbone *et al.*, 1971), and used to evaluate prognosis. Other radiological imaging is used as clinically indicated e.g. magnetic resonance imaging (MRI) of the brain and/or spinal cord or bowel.

Due to the non-specific clinical presentation of PTLT and the assumption that early diagnosis and treatment improves overall survival, many studies have used EBV load to monitor patients at high risk of developing PTLT. However many variables exist in

the published methods used to measure EBV load, confounding the data. These include: (a) the amplification techniques e.g. semi-quantitative (SC-), quantitative-competitive (QC-), real time quantitative (RTQ-) and light cycler quantitative (LCQ-) forms of PCR; (b) the peripheral blood component used as test samples e.g. whole blood, PBMC, serum or plasma; (c) the means of expressing EBV copy number e.g. per ml whole blood, μ g DNA, numbers of lymphocytes and (d) no international standard for EBV has yet been established so laboratories tend to use their own. In addition, an important conceptual problem with measuring the EBV load in blood samples is that the virus is exclusively present in the resting memory B cell population or shed from the lytic cycle of these cells into plasma or serum and does not reflect EBV in the PTLD tumour (Babcock *et al.*, 1999). Therefore measurement of the viral load in the blood will represent the degree of immune deficiency allowing viral expansion secondary to immunosuppression and not events leading to transformation to PTLD in the tissues.

The clinical studies addressing the use of viral load in the diagnosis of PTLD are difficult to interpret because they mostly involve small numbers of patients and, starting with the assumption that a high EBV load will predict development of PTLD, most of the studies incorporated pre-emptive therapeutic intervention when EBV levels reached certain levels (Savoldo *et al.*, 2005; Holmes *et al.*, 2002; Lee *et al.*, 2005; McDiarmid *et al.*, 1998). There is a preponderance of paediatric studies because of the increased risk of PTLD in EBV sero-negative individuals undergoing transplantation.

The most consistent findings are seen in HSCT patients where the risk of PTLD is largely restricted to the first 6 months posttransplant, during which the immune

repertoire is being generated. A combination of EBV PCR and measurement of EBV specific T cells by means of tetramers has helped to define patients at risk of PTLD and those at risk were then treated pre-emptively with rituximab to eliminate EBV infected B cells during the time required to regain the immune repertoire (Clave *et al.*, 2004). Most studies in HSCT patients agreed on a cut-off value of $5 \times 10^3 - 10^5/\mu\text{g}$ DNA as carrying a high risk of PTLD and there is a consensus that a rapid, progressive rise in EBV load is the most important indicator of PTLD (Clave *et al.*, 2004; Gartner *et al.*, 2002; Orentas *et al.*, 2003). Indeed, one group noted that in patients developing PTLD, the EBV load a week earlier was below the cut-off value indicating the need for frequent estimation of EBV load (Gartner *et al.*, 2002). Successful treatment with rituximab led to a prompt fall in EBV load. Encouraging though these studies are, they do not equate to solid organ grafts because the presence of tetramer positive EBV specific cytotoxic T cells does not imply that they are functional when immunosuppressive drugs are used. In HSCT immunosuppression is light and often not required; hence the presence of tetramer positive cells is synonymous with functional CD8^+ T cells.

Studies measuring EBV load in solid organ transplantation are more controversial and discordant than for HSCT. Cut-off levels for the risk of PTLD ranged between 200 and $500/10^5$ PBMC (Green *et al.*, 1998; Gridelli *et al.*, 2000), 600 and $2.5 \times 10^4/\mu\text{g}$ DNA (Leung *et al.*, 2004; Savoldo *et al.*, 2005) or $10^4/\text{ml}$ (Benden *et al.*, 2005). Some groups even set separate cut-off values for EBV sero-negative ($>40/10^5$ PBMC) and sero-positive ($>200/10^5$ PBMC) recipients (Green *et al.*, 1998; Holmes *et al.*, 2002). A meta-analysis of studies using a cut-off value to predict PTLD showed that 24/135 (18%) of patients with high EBV levels actually developed PTLD (Gridelli *et al.*,

2000; Kullberg-Lindh *et al.*, 2006; Scheenstra *et al.*, 2004; Vajro *et al.*, 2000; Benden *et al.*, 2005; Merlino *et al.*, 2003; Green *et al.*, 2000). In studies where intervention was not used the general conclusion was that EBV load could not accurately predict the development of PTLN (Scheenstra *et al.*, 2004; Vajro *et al.*, 2000) and some concluded that the EBV load was more value as a negative predictor of PTLN for patients whose EBV was low or negative (Allen *et al.*, 2001; Green *et al.*, 2000). However, in the studies that did include therapeutic intervention, some benefit was observed against historical controls as a result of reducing immunosuppression or other therapeutic manoeuvres (Lee *et al.*, 2005; McDiarmid *et al.*, 1998). Other studies improved the predictive power of EBV load by including an assessment of the immune response to EBV (Smets *et al.*, 2002; Carpentier *et al.*, 2003; Baudouin *et al.*, 2004).

Therefore, until randomised studies are performed, the predictive value of EBV load for anticipating development of PTLN following SOT remains uncertain.

1.4.4 Prognosis

Reported rates of disease-free and overall survival in PTLN are variable due to the heterogeneity of the disease. Estimated survival rates range from 25%–60% (Leblond *et al.*, 2001). A recent study of 107 cases proposed a multivariate prognostic model in which poor performance status, monomorphic disease, and grafted organ involvement predicted poor outcome (Ghobrial *et al.*, 2005). Other negative risk factors that have been implicated include patients who are EBV sero-negative at the time of transplant, late onset of disease, disease involving multiple sites, advanced stage, elevated lactate

dehydrogenase (LDH), severe organ dysfunction, and CNS disease (Tsai *et al.*, 2001; Choquet *et al.*, 2002; Benkerrou *et al.*, 1998; Trofe *et al.*, 2005).

1.4.5 Treatment

The treatment of PTLT is controversial with no international consensus. To date no randomised multi-centre trials have been performed and published studies typically reflect the retrospective experience of single institutions. The basic principle of treatment however, is to stop uncontrolled EBV-driven B cell proliferation; either by restoring effective EBV-specific T cell immunity or by targeting the malignant B cells with anti-B cell antibodies (rituximab) or cytotoxic chemotherapy. Differences exist in the treatment of PTLT following SOT or HSCT and will be considered separately.

1.4.5.1 Treatment of established PTLT following SOT

Reduction of immunosuppressive therapy: Since this strategy was first described in 1984 to treat PTLT (Starzl *et al.*, 1984) it has been the first line treatment of PTLT occurring after SOT and is used in over 90% of cases (Amlot *et al.*, 2007). Following SOT patients require a combination of major immunosuppressive drugs to control against alloreactivity and graft rejection. Immunosuppression however, has the adverse effect of reducing the number of functional circulating EBV-specific T cells leading to uncontrolled EBV B-cell proliferation. The rationale for reducing immunosuppression is therefore to restore EBV immunity allowing the EBV-specific cytotoxic T cells to kill the malignant B cells. Studies have shown that this approach is effective in approximately 30-50% of all cases (Starzl *et al.*, 1984; Amlot *et al.*, 2007). At present there is no standard algorithm for reduction in immunosuppression

(RIS), however, it is important that the patient is monitored closely throughout for deterioration in graft function (indicative of rejection) or in clinical status.

A novel approach offered as a clinical service in our centre, is to reduce the major immunosuppressive drugs (e.g. cyclosporine, tacrolimus) in a step-wise fashion tailored to the patient's clinical performance status according to the Karnofsky scale (**Appendix 1.2**), the size of the target PTLT tumour used to assess clinical response and the re-emergence of an anti-viral response by immunophenotyping the patients T cells for activation markers such as surface MHC Class II expression (Amlot *et al.*, 1996; Amlot *et al.*, 2007). This approach is illustrated in **Table 1.4** and has the advantage of identifying those patients with life threatening PTLT who require urgent treatment and rapid RIS. Conversely, it also identifies patients with stable disease who can have their immunosuppression tapered more cautiously. At each step of RIS, patients are assessed for an increase in T cell activation indicative of an anti-viral response. Immunosuppression continues to be reduced at a rate determined by the patient's clinical status (**Table 1.4**) until there is a three fold rise in the number of MHC class II expressing CD8⁺ T cells to achieve at least $0.3 \times 10^9/L$ (Amlot *et al.*, 2007). RIS is also stopped if the target PTLT tumour decreases in size by 30% according to the RECIST criteria (**Appendix 1.3**), indicating a partial clinical response, or if graft rejection occurs.

By using this tailored individual approach, PTLT patients are less likely to have graft rejection, a scenario which is clinically devastating especially for heart, lung and liver transplant patients, where replacement allografts are rare and no effective support therapy (such as haemodialysis for failed renal allografts) exists.

Week	% of the starting immunosuppressive dose at PTL D diagnosis ¹		
	Stable PTL D ²	Progressive PTL D ³	Life threatening PTL D ⁴
0	90	90	60
1	90	75	
2	75	60	30
3	75	45	
4	60	30	0
5	60	12	
6	45	0	
7	45		
8	30		
9	30		
10	15		
11	15		
12	0		

Table 1.4: Protocol for RIS

Depending on the clinical status it is possible for a patient to move categories, e.g. if a patient had progressive disease that became stable at week 3, then subsequent reduction carries on from the dose achieved (45%) at fortnightly intervals in the stable disease column.

¹ Immunosuppressive regimes usually include a combination of azathioprine (AZA) or mycophenylate mofetil (MMF) plus Cyclosporine (CSA) or tacrolimus (TAC) with or without steroids. At PTL D diagnosis AZA and MMF are stopped and the sliding scale of RIS usually refers to CSA or TAC. The exception is where the immunosuppressive regime at diagnosis is AZA or MMF or rapamycin in combination with steroids. In this case the drug being reduced according to the sliding scale is either AZA or MMF or rapamycin. The dose of steroids remains unchanged.

² Stable PTL D: No change in the size of measurable PTL D lesions and where the patient's clinical status according to the Karnofsky scale (**Appendix 1.2**) is unchanged.

³ Progressive PTL D: 20% increase in the size of measurable PTL D lesions or a deterioration in the patient's clinical status according to the Karnofsky scale as a direct result of the PTL D. Patient remains in Karnofsky grade $\geq 20\%$.

⁴ Life threatening PTL D: PTL D is affecting the vital organs (heart, lungs circulatory system, graft) and the patient is moribund (Karnofsky grade 10%).

Chemotherapy: Combination chemotherapy regimes that are typically used for NHL (e.g. cyclophosphamide, doxorubicin, vincristine and prednisolone, CHOP) are often used in the treatment of PTLT when RIS fails to control the disease (Garrett *et al.*, 1993; Muti *et al.*, 2002). Although combination chemotherapy is effective, treatment-related mortality rates secondary to neutropenic sepsis are high. Recent studies however, have shown that this may be reduced by using low-dose chemotherapy regimes such as cyclophosphamide and prednisolone (Gross *et al.*, 2005; Gross *et al.*, 1998).

Surgery and Radiation: Localised PTLT may be treated with a combination of RIS and surgery (Beynet *et al.*, 2004) or radiotherapy (Koffman *et al.*, 2000). These modalities are particularly important where disease is causing compression of vital organs and urgent relief is required.

Anti-viral agents: B cell proliferation in PTLT is associated with the latent cycle of EBV infection and is independent of lytic replication. Therefore anti-viral drugs such as acyclovir or ganciclovir which prevent lytic viral replication have no clinical value in the treatment of established PTLT. Some transplant centres use prophylactic anti-viral therapy in EBV sero-negative patients who receive a sero-positive graft in an attempt to limit viral transmission from the donor to the recipient; however the evidence to support such management is lacking. Babcock *et al* showed that in asymptomatic EBV sero-positive organ transplant recipients with elevated viral loads, the EBV load was maintained in the resting memory B cell population (Babcock *et al.*, 1999) and therefore speculated that anti-viral therapy would have limited, if any, effect on the prevention of PTLT. This is supported by a randomised trial which showed no reduction in the incidence of PTLT with prolonged acyclovir therapy

(Green *et al.*, 1997). Other retrospective clinical studies have shown some benefit with anti-viral prophylaxis, however they are limited by the lack of contemporaneous controls and patient numbers (Funch *et al.*, 2005; Darenkov *et al.*, 1997; Davis *et al.*, 1995).

Cytokine and anti-cytokine therapy: IFN- α has been used in the treatment of PTLT with some clinical success (Davis *et al.*, 1998), however an increased incidence of graft rejection was also reported and therefore this therapy should be used with caution. Antibodies against IL-6, the cytokine involved in the proliferation and maturation of B cells, have also been trialled as a novel treatment of PTLT which is unresponsive to RIS. In a multi-centre phase I/II trial of 12 SOT PTLT patients, treatment with a murine anti-IL-6 monoclonal antibody produced clinical remissions in 5 patients and partial responses in a further 3 patients. No relapse was observed in a 15-27 month follow-up period (Haddad *et al.*, 2001). Importantly, these studies used abrupt cessation of immunosuppression as the initial RIS schedule, and if no response had occurred after 8 days patients were assumed unresponsive and eligible for the study. Patients in this study may therefore not fully represent the poor prognostic group of PTLT patients that are unresponsive to RIS and are more likely respond to further immune manipulation. Further trials are required to confirm this beneficial effect.

Anti-B cell antibodies: Rituximab, a chimeric monoclonal antibody that targets CD20 on the surface of B cells has been used in several small retrospective studies as a treatment for PTLT following SOT (Milpied *et al.*, 2000; Cook *et al.*, 1999; Caillard *et al.*, 2000; Zompi *et al.*, 2000; Yang *et al.*, 2000b). Typically Rituximab is used as a second-line therapy after RIS has failed (defined as patients who remain unresponsive

to RIS after a 7-14 day period), either alone or in conjunction with chemotherapy. Response rates from the above studies varied (20-100%), emphasising the difficulties in assessing rituximab from small and heterogeneous patient populations. Recently, however a prospective multi-centre phase II trial of 46 SOT patients who had failed to respond to withdrawal of immunosuppressive therapy showed an overall response rate of 46% and an overall 1 year survival rate of 67% (Choquet *et al.*, 2006). Whether this response rate could be improved by using rituximab in conjunction with combination chemotherapy (e.g. CHOP regime), a strategy which has been used effectively in immuno-competent NHL patients (Coiffier *et al.*, 2002), has yet to be evaluated in clinical trials. Although rituximab is a promising therapy in patients who fail to respond to RIS, it has several potential disadvantages. Firstly as rituximab is specific for CD20, it is possible that its use on PTLD tumours that have heterogeneous CD20 expression may cause the outgrowth of a CD20-negative population of proliferating B cells. This phenomenon has been previously reported in a few NHL patients (Kennedy *et al.*, 2002; Davis *et al.*, 1999) but as yet has not been seen in PTLD. Secondly, it is unclear what effect the profound B cell depletion caused by rituximab (B cells are depleted for 6-8 months post treatment) has on patients who are already immunosuppressed following transplantation (Suzan *et al.*, 2001).

Cellular Immunotherapy: A novel means of restoring EBV-specific cellular immunity in patients with PTLD is to infuse EBV-specific CTLs that are generated *ex vivo*. PTLD following SOT is invariably of host origin and therefore CTLs must either be expanded from autologous or closely HLA-matched donor PBMCs. Autologous EBV-specific CTLs have been piloted as a prophylactic treatment for PTLD in two small studies with some clinical response (Comoli *et al.*, 2002; Savoldo *et al.*, 2006).

This approach however, is limited by the failure of standard techniques to generate CTLs from EBV sero-negative patients; a group who have an increased risk of developing PTLN following transplantation of a sero-positive organ, and the prohibitive time that is required to generate the CTLs (2-3 months). One means of addressing these problems is to develop a bank of cryopreserved allogeneic EBV-specific CTLs from the blood of healthy EBV sero-positive donors. CTLs can then be selected from a panel of lines according to the HLA type of the patient and used immediately (Wilkie *et al.*, 2004; Haque *et al.*, 2002; Haque *et al.*, 2001). A recent phase I/II clinical trial used allogeneic EBV-specific CTLs matched on HLA-A, -B and where possible -DR to treat eight patients with EBV-positive PTLN that had failed to respond to treatment with RIS +/- rituximab (Haque *et al.*, 2002). Prior to infusion the high specificity for EBV antigens and low specificity for alloantigens was confirmed by testing the CTLs in *in vitro* cytotoxicity assays against the autologous EBV-immortalised LCL used to generate the CTL line, the patients phytohaemagglutinin-blasts or the patients LCL (where available), a HLA-mismatched LCL and a NK cell line. Clinical responses were observed in 4 out of 8 patients, however two died from causes unrelated to CTL toxicity before assessment of clinical response was possible. Although these responses are encouraging, this approach is limited by the short-term persistence of the CTLs (EBV-specific precursors were only detected by limiting dilution analysis (LDA) for a maximum of 44 days) and may be best suited to patients with low volume disease. Another criticism of this study is that the time interval from RIS +/- rituximab to infusion of EBV-specific T cells was short, suggesting that the patients had not necessarily failed to respond to first-line therapy.

Vaccination: As EBV sero-negative patients are at high risk of developing PTLD after receiving a sero-positive graft, one approach to reduce the incidence of PTLD is to vaccinate all patients prior to transplantation and induce EBV sero-conversion. A vaccine containing the gp350 viral envelope antigen previously demonstrated a good immunological response in healthy EBV sero-negative healthy volunteers (Gu *et al.*, 1995) and is currently being piloted in EBV sero-negative children awaiting transplant. The primary endpoint of this feasibility study however, is to assess the safety and optimal dose of the vaccine and is not powered to show whether the vaccine prevents PTLD.

1.4.5.2 Treatment of established PTLD following HSCT

The differences in treatment between PTLD following SOT and HSCT relate to the means by which these patients become immunosuppressed. Following SOT, patients are treated with a combination of major immunosuppressive drugs to control against graft alloreactivity. Immunosuppression is profound and indicated for life. In HSCT patients however, the immunodeficiency is caused by the ablation of the patient's immune system with a combination of high dose chemotherapy and/or radiation prior to donor stem cell infusion and recovers with immune reconstitution of the donor graft. Immunosuppressive drugs are not usually required following HSCT and RIS is therefore rarely used as a therapeutic strategy. Instead, treatment is directed at reduction of the B cell mass with anti-B cell antibodies or restoring EBV T cell immunity with adoptive transfer of donor EBV-specific T cells. Cytotoxic chemotherapy in these patients has the potential to induce graft failure or prolonged pancytopenia and is therefore avoided.

Anti-B cell antibodies: Unlike SOT patients, the efficacy of Rituximab has not been assessed in a multi-centre phase II trial. However, several small case studies have reported complete response rates ranging from 66 to 100% (McGuirk *et al.*, 1999; Milpied *et al.*, 2000; Kuehnle *et al.*, 2000; Faye *et al.*, 2001; van Esser *et al.*, 2001). Rituximab was used in conjunction with irradiated donor T cells or donor-derived EBV-specific CTLs in two of these studies (McGuirk *et al.*, 1999; van Esser *et al.*, 2001), thus confounding the data.

The drawbacks of rituximab as indicated for SOT also apply to its use in HSCT patients.

Cellular Immunotherapy: Donor-derived EBV-specific T cells have been used with clinical success in several studies as both prophylaxis and treatment for established PTLD in HSCT recipients (Gustafsson *et al.*, 2000; Rooney *et al.*, 1995; Rooney *et al.*, 1998b; Weinstock *et al.*, 2006). PTLD tumours following HSCT are typically of donor origin and therefore EBV-specific CTLs are generated from donor blood using donor-derived EBV-transformed LCLs as APCs. Donor lymphocytes are readily available following HSCT and as donors are adults the majority will be EBV sero-positive. EBV-specific CTLs still require 2-3 months to prepare and validate however, and therefore would need to be prepared prior to transplant from each donor. Allogeneic EBV-specific T cells represent an alternative however this has only been trialled in one HSCT patient (Haque *et al.*, 2002).

1.5 Clinical management of EBV-positive HD

EBV-positive HD represents 40-50% of all HD cases although this figure increases in developing countries and in patients with coexistent HIV infection. EBV-positive and EBV-negative tumours are currently managed according to the same treatment algorithms. However, as further information regarding the role of EBV in the pathogenesis of HD becomes available, novel treatment strategies for EBV-positive HD may be developed. Current interest is directed at the use of the EBV antigens as targets for cellular immunotherapy and this will be discussed in detail below.

1.5.1 Incidence

Many studies have suggested a causative association between EBV and HD (Gutensohn and Cole, 1980; Chatila *et al.*, 1989; Weiss *et al.*, 1987; Wu *et al.*, 1990) however, the precise mechanisms involved remain unclear. The main premise for an infectious origin for HD is the distinct pattern of incidence within age-groups. The incidence of HD is traditionally described as bimodal, with a peak incidence in childhood (developing countries) or early adulthood (developed countries) followed by a second peak in middle age. The early peak mirrors the age at which primary EBV infection occurs suggesting that HD might develop from an atypical response to EBV. Against this however, is that the majority of HD tumours occurring in young adults (who have the highest incidence of IM) are not associated with EBV. A recent population study has proposed that the bimodal incidence model is over simplistic and that four distinct types of HD occur at different ages and geographical areas (**Figure 1.9**). The first three represent EBV-positive HD, while the fourth represents EBV-negative HD and accounts for the large incidence in young adults from developed

countries (Jarrett, 2002). It is therefore very possible that the small peaks of EBV-positive HD at an early age do represent delayed reactions to primary EBV infection.



Figure 1.9: Incidence of HD by age

The coloured lines represent three distinct forms of EBV-associated HD cases. The first (**a**) occurs below the age of 10 years and accounts for most childhood cases of HD in developing countries. The second (**b**) represents EBV-associated HD among young adults and has a peak incidence between 15-34 years. Both forms of HD are thought to represent a delayed atypical reaction to primary EBV infection. The third type of EBV-associated HD (**c**) has a peak incidence in middle age and represents over 50% of cases over the age of 55 years. The dotted line (**d**) represents the cases of HD that are not associated with EBV which account for the largest incidence among young adults in developed countries.

Figure adapted from (Jarrett, 2002).

1.5.2 Clinical presentation

Patients with HD typically present with painless lymphadenopathy. Other features may include the so-called 'B' symptoms: unexplained fevers with temperatures over

38°C, drenching night sweats and unexplained loss of more than 10% of body weight in the 6 months before diagnosis. Pruritus and fatigue are also commonly described.

1.5.3 Diagnosis

EBV-positive HD is diagnosed from histopathological examination of an affected lymph node. The characteristic R-S cells are identified as large often binucleated cells with prominent nucleoli and an unusual CD45-, CD30+, CD15+/- immunophenotype. The presence of EBV is confirmed by nuclear staining for EBERs using *in situ* hybridization and LMP cell surface expression detected by immunohistochemical staining.

Following diagnosis, the patient's disease is staged using radiological imaging and bone marrow aspiration and classified according to the Ann Arbor staging system (**Appendix 1.1**, (Carbone *et al.*, 1971).

1.5.4 Prognosis

The prognosis of HD can be accurately determined using a series of seven prognostic factors determined from a large international study (Hasenclever and Diehl, 1998). These include: age > 45 years, stage IV disease, male sex, haemoglobin < 10.5 mg/dl, lymphocyte count $\leq 0.6 \times 10^9/L$, albumin < 4.0 mg/dl and white blood count $\geq 15 \times 10^9/L$. Freedom from progression (FFP) at 5 years directly relates to the number of adverse prognostic factors present in each patient. The 5 year FFP for patients with no adverse factors is 84%. Each additional factor lowers the 5 year FFP by 7%, such that a patient with 5 or more factors has a 5 year FFP of only 42%.

Three recent population-based cohort studies have also shown the presence of EBV to be a poor prognostic factor, but only in patients > 45 years (Keegan *et al.*, 2005a; Enblad *et al.*, 1999; Jarrett *et al.*, 2005). Whether this observation is a consequence of two biologically distinct forms of HD or the decline in EBV-specific cellular immunity with age is unclear.

1.5.5 Treatment

The treatment of HD has changed significantly over the last decades, rendering this entity one of the most curable human cancers. Currently, approximately 80% of patients achieve long-term disease-free survival. Progress has been made by tailoring poly-chemotherapy regimes that not only induce high rates of remission but have a reduced incidence of fatal secondary malignancies. The prognosis of patients who fail to achieve remission with primary treatment (Primary progressive disease) or who developed relapsed disease however remains poor, necessitating research into novel therapeutic strategies.

1.5.5.1 Early Stage HD

Traditionally stage I or II HD was treated with radiotherapy which extended beyond the sites of disease (extended-field radiation). However due to the high incidence of relapse and the significant long term morbidity and mortality from cardiac toxicity, this approach has been superseded by the use of 2-4 cycles of combination chemotherapy regimes such as doxorubicin, bleomycin vinblastine and dacarbazine; ABVD, in conjunction with radiotherapy to the involved nodes only (involved-field radiation). Patients with unfavourable risk factors (Large mediastinal mass, age \geq 50

years, high erythrocyte sedimentation rate or extra-nodal disease) are treated with 4-6 cycles of ABVD +/- involved-field radiation.

1.5.5.2 Advanced disease

Patients with stage III or IV HD are typically treated with 6-8 cycles of ABVD combination chemotherapy. This strategy has demonstrated overall survival rates of between 65-82% in two multi-centre international phase III trials (Duggan *et al.*, 2003; Canellos and Niedzwiecki, 2002; Canellos *et al.*, 1992). Recent trials have shown that survival figures can be further improved by using dose-intensified chemotherapy regimes (Horning *et al.*, 2002; Diehl *et al.*, 2003), however at the expense of increased secondary haematological malignancy rates (Diehl *et al.*, 2003). The role of consolidating radiotherapy after effective chemotherapy in the treatment of advanced disease remains controversial. A large phase III trial and a meta-analysis of several trials showed no clinical benefit of consolidative involved-field radiotherapy after achieving a complete remission with combination chemotherapy (Aleman *et al.*, 2003; Loeffler *et al.*, 1998), although some benefit was observed in patients achieving a partial remission (Aleman *et al.*, 2003).

1.5.5.3 Primary progressive and relapsed Hodgkin's disease

Patients with primary progressive disease or who relapse after achieving complete remission with chemotherapy may be salvaged with high dose chemotherapy in conjunction with autologous HSCT (Linch *et al.*, 1993; Schmitz *et al.*, 2002). However, many patients will relapse again after this approach and require further treatment (Disease-free survival at 3 yrs 75% (Linch *et al.*, 1993) and 55% (Schmitz *et al.*, 2002) in the transplant arms of both randomised control trials).

Allogeneic HSCT with reduced intensity conditioning has been trialled with some clinical success by several centres for the treatment of multiple relapsed or primary progressive disease (Alvarez *et al.*, 2006; Anderlini *et al.*, 2005; Peggs *et al.*, 2005), indicating that a graft-versus-lymphoma effect may exist. The short follow-up period for these patients to date however, limits true assessment of the impact on progression-free and overall survival. In addition, treatment related mortality (TRM) is high (4-17% at day 100) with this approach restricting its utility. One mechanism by which graft-versus-lymphoma may be mediated in EBV-positive HD patients is by the EBV-latent antigens (LMP1, LMP2) acting as tumour-associated antigens for EBV-specific CTLs. Donor-derived EBV-specific CTLs are known to be transferred via allografts from EBV sero-positive donors and require persistent EBV antigen for maintenance before EBV-specific CTLs can be generated in the recipient after immune reconstitution (Gandhi *et al.*, 2003). Whether this effect contributes to an improved clinical outcome for EBV-positive HD after allogeneic HSCT compared to EBV-negative HD has yet to be assessed.

Donor lymphocyte infusions (DLI) have also been used for residual disease or relapse after allogeneic HSCT (Peggs *et al.*, 2005; Anderlini *et al.*, 2004; Alvarez *et al.*, 2006), again with some clinical response. This success however, is balanced by a high incidence of GVHD (33-88%) and TRM (11-33%).

Unlike non-Hodgkin's lymphoma where humanised monoclonal antibodies are used extensively, their use in HD is still undefined and subject to further research. Bi-specific antibodies against CD30, an antigen on R-S cells and CD16, an antigen on NK cells have been developed to induce tumour directed cytotoxicity and used in a small phase I/II trial of heavily treated refractory HD patients with a 25% response

rate (Hartmann *et al.*, 2001). Similar results were also achieved with another bi-specific antibody against CD30 and CD64 (Borchmann *et al.*, 2002), however both studies were hindered by the development of anti-murine antibodies. Studies are currently in progress using a fully humanised antibody to CD30 (Borchmann *et al.*, 2003; Boll *et al.*, 2005).

A novel approach restricted to the treatment of relapsed EBV-positive HD is adoptive transfer of EBV-specific CTLs. Early studies used CTLs that had been generated *in vitro* from repeated stimulation with autologous LCLs which preferentially stimulate T cells specific for EBNA antigens rather than the LMP 1 and 2 antigens expressed by R-S cells (Rooney *et al.*, 1998a; Roskrow *et al.*, 1998; Bollard *et al.*, 2004). Despite this broad specificity, however, some clinical responses were demonstrated and using tetramer and functional analyses it was possible to track LMP2-specific CTLs entering the tumours (Bollard *et al.*, 2004). More recent studies have used a recombinant vaccinia-virus polyepitope construct that encodes for multiple LMP1 and 2 epitopes to generate LMP-specific CTLs in healthy individuals (Duraismamy *et al.*, 2004; Duraismamy *et al.*, 2003). This strategy has yet to be used in patients with HD and has the disadvantage of being technically demanding and extremely time consuming. Treating multiple patients with LMP-specific CTLs is therefore unlikely to be a realistic future treatment option. One way to circumvent these disadvantages is to use allogeneic EBV-specific CTLs from partially HLA-matched donors; an approach described above for the treatment of PTLN (Haque *et al.*, 2002). To date this has been studied in the context of case reports only (Bollard *et al.*, 2006; Lucas *et al.*, 2004).

1.6 Aims of the thesis

To describe the development a flow cytometry based assay for the rapid enumeration of functional EBV-specific CD8⁺ T cells in PBMCs using production of interferon- γ as a readout.

- The assay was optimised in healthy EBV sero-positive individuals testing a variety of different EBV antigens to stimulate responses and then applied to patients with two EBV-driven malignancies: PTLN and HD.
- In patients with PTLN, the assay was used to monitor the emergence of EBV immunity in patients whose immunosuppressive therapy was reduced, and recovery of functional EBV immunity investigated for correlation with clinical outcome and compared with current immunophenotyping techniques used to guide RIS.
- In patients with EBV-positive HD, the assay was used to investigate the role of EBV-specific CD8⁺ and CD4⁺ T cell responses in the pathogenesis of this disease.

To describe the development of a novel approach for detecting and quantifying EBV-specific T cell responses using real-time quantitative PCR to measure cytokine production. This technique was compared to the flow cytometry-based assay using samples from healthy individuals and patients with EBV-driven malignancies.

To describe the investigation of whether the presence of EBV in pathological tissues can be accurately detected using a novel murine antibody, RFD3. Results were compared to the current detection methods used in clinical practice.

Chapter 2

Materials and Methods

2.1 Peptide Biochemistry

2.1.1 Source

The amino acid sequences of previously identified EBV peptides bound by HLA class I molecules and recognised by T cells were retrieved from the SYFPEITHI database (<http://syfpeithi.de>). EBV peptides were produced on an ABI synthesiser using Fmoc chemistry and purified by high performance liquid chromatography by Yorkshire Bioscience, Heslington, York, UK (<http://york-bio.com>). All peptides were produced to greater than 95% purity and expected molecular weight was confirmed by mass spectrometry. Some HLA-A*0201 binding EBV peptides were also produced at 80% purity. Peptides were reconstituted in dimethyl sulfoxide (DMSO, Sigma, Surrey, UK) to give stock solutions of 100mg/ml, 20mg/ml and 10mg/ml (weight:volume), and stored at -70°C.

2.1.2 EBV specific peptides

The EBV peptides used are listed in **Tables 2.1 and 2.2**. Peptides were chosen to cover a broad spectrum of HLA alleles.

<i>Peptide Sequence</i>	<i>Protein</i>	<i>HLA restriction</i>
CLGGLTMTV	LMP 2	HLA-A *0201
AVFDRKSDAK	EBNA 3B	HLA-A *0201
LLDFVRFMGV	EBNA 3C	HLA-A *0201
GLCTLVAML	BMLF 1	HLA-A *0201
LLWTLVVLL	LMP 2	HLA-A *0201
SVRDRLARL	EBNA 3A	HLA-A *0201
LMHIPLINV	gp85	HLA-A *0201
TLFIGSHVV	gp85	HLA-A *0201
SLVIVTTFV	gp85	HLA-A *0201
VLQWASLAV	gp350	HLA-A *0201
LLWAARPRL	BARFO ORF	HLA-A *0201
YLLEMLWRL	LMP 1	HLA-A *0201
SLREWLLRI	EBNA 3A	HLA-A *0203
LLSAWILTA	LMP 2	HLA-A *0203
LTAGFLIFL	LMP 2	HLA-A *0206
RLRAEAQVK	EBNA 3C	HLA-A *03
AVFDRKSDAK	EBNA 3B	HLA-A *1101
IVTDFSVIK	EBNA 3B	HLA-A *1101
SSCSSCPLSK	LMP 2	HLA-A *1101
ATIGTAMYK	Rta	HLA-A *1101
PYLFWLAAI	LMP 2	HLA-A *23
DYCNVLNKEF	Rta	HLA-A *24
RYSIFFDY	EBNA 3C	HLA-A *24
TYGPVFMCL	LMP 2	HLA-A *24
AYSSWMYSY	EBNA 3C	HLA-A *3002
AVFDRKSDAK	EBNA 3B	HLA-A *6801

Table 2.1: EBV peptide sequences presented by HLA-A

<i>Peptide Sequence</i>	<i>Protein</i>	<i>HLA restriction</i>
VPAPAGPIV	EBNA 3A	HLA-B *07
QPRAPIRPI	EBNA 3C	HLA-B *0702
RPPIFIRRL	EBNA 3A	HLA-B *0702
FLRGRAYGL	EBNA 3A	HLA-B *08
QAKWRLQTL	EBNA 3A	HLA-B *08
RAKFKQLL	BZLF 1	HLA-B *08
AYPLHEQHG	EBNA 3A	HLA-B *08
YIKSFVSDA	EBNA 3A	HLA-B *08
QNGALAINTF	EBNA 3C	HLA-B *1501
LEKARGSTY	EBNA 3A	HLA-B *1501
GQGSPTAM	EBNA 3B	HLA-B *1501
DEVEFLGHY	BMLF 1	HLA-B *18
HPVGEADYFEY	EBNA 1	HLA-B *3501
AVLLHEESM	EBNA 3B	HLA-B *3501
YPLHEQHGM	EBNA 3A	HLA-B *3503
IEDPPFNSL	LMP 2	HLA-B *4001
SENDRLRLL	BZLF 1	HLA-B *4002
VEITPYKPTW	EBNA 3B	HLA-B *4402
KEHVIQNAF	EBNA 3C	HLA-B *4402
EENLLDFVRF	EBNA 3C	HLA-B *4402
AEGGVGWRHW	EBNA 3C	HLA-B *4403
VSFIEFVGW	EBNA 3B	HLA-B *5801
YRSGIIAVV	BMLF 1	HLA-Cw *06

Table 2.2: EBV peptide sequences presented by HLA-B and HLA-Cw

2.2 *In vitro* Cell Culture

2.2.1 Blood sample collection

Blood from patients with PTLD or HD was received from five renal transplant centres: The Oxford Kidney Unit, The Churchill Hospital, Oxford; Gloucester Royal Hospital, Gloucester; Wessex Renal Transplant Unit, Queen Alexandra Hospital, Portsmouth; St Bartholomew's Hospital, London and The Royal Free Hospital, London. Ethical permission was granted locally for each participating centre.

Approximately 30mls of blood in Lithium heparin tubes was taken at the same time routine samples were collected for immunophenotyping of lymphocyte activation subsets, a clinical service provided by the Clinical Immunology Department at the Royal Free and University Medical School (RFUCMS), London.

EBV sero-positive healthy volunteers from the Anthony Nolan Research Institute and the Immunology Department of the Royal Free Hospital were recruited and consented to participate in this study.

HLA genotyping for Class I and II HLA alleles (HLA-A, B, Cw and DR, DP and DQ) for healthy individuals was performed by the histocompatibility laboratories of the Anthony Nolan Trust (ANT). HLA typing for the patient group was performed locally at the transplant centres using a combination of low and high resolution techniques.

2.2.2 Media and Reagents

All media and supplements were obtained from BioWhittaker, Wokingham, UK, unless otherwise stated. Plastic ware was obtained from Elkay, Basingstoke, UK, or Helena Biosciences, Sunderland, UK.

2.2.3 Cell culture conditions

Cell lines (described below) and peripheral blood mononuclear cells (PBMCs) were grown in complete media (CM: RPMI 1640 media supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, and 10% heat-inactivated foetal calf serum), in a 37°C humidified incubator in 5% CO₂. Cells were grown at a density of $0.5-1 \times 10^6/\text{ml}$ in 25cm² or 50cm² tissue culture flasks (Falcon™, Becton Dickinson (BD), Oxford, UK). CM was replaced every three days or earlier if the

phenol red in the media had turned yellow indicating acidity. Sterile conditions were maintained by working in a category II microflow cabinet.

2.2.4 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood

Whole blood was collected into lithium heparin (Starstedt, Germany) tubes to prevent clotting. A 4ml aliquot of whole blood was carefully layered onto 4ml Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 2200 rpm for 20 minutes. PBMCs were collected from the interface using a wide bore sterile pastette, washed in RPMI 1640 media and centrifuged at 1500 rpm for 7 minutes. The cell pellet was washed in CM, centrifuged at 1200 rpm for 5 minutes, resuspended in CM, and counted in a Neubauer haemocytometer and adjusted to a concentration of $1 \times 10^6/\text{mL}$. PBMCs were used immediately or cryopreserved for later experiments.

2.2.5 Whole blood preparation

Whole blood was transferred from the lithium heparin collection tubes into 15ml conical tubes and centrifuged at 2200 rpm for 10 minutes. Serum was removed with a pastette and replaced with an equivalent volume of CM. Red cells were lysed using sterile Hoffman's lysis buffer (8.3g NH_4Cl , 1.0g KHCO_3 , 37mg disodium EDTA dissolved in 100ml distilled water, pH 7.6) diluted 1:10 with distilled water, by adding 1ml of serum-free whole blood to 14ml of diluted Hoffman's lysis buffer and incubating at room temperature for 5 minutes. After centrifugation the cells were washed immediately and resuspended in CM equivalent to the original volume of whole blood.

2.2.6 Single cell suspension from lymph node biopsies

Single cell suspensions were prepared from fresh lymph node biopsies that had been taken at the Department of Clinical Immunology, Royal Free Hospital after receiving ethical permission. Lymph nodes were placed into a Petri dish with CM and cut up into small pieces using a sterile scalpel. The tissue pieces were then placed into a 50ml conical tube with CM and further disintegrated by gently pipetting. The tissue pieces and the CM were gently passed through a sieve into a 50ml conical tube, generating a cell suspension. The debris was allowed to settle for 2 minutes and the single cell suspension was transferred to a clean tube for counting.

2.2.7 Cell counting and viability

Cells were counted using a 0.1mm depth Neubauer haemocytometer (Weber Scientific International, West Sussex, UK). A 10 μ L aliquot was taken from a known volume of cells resuspended in CM and mixed thoroughly with a 10 μ L aliquot of 0.4% Trypan Blue solution (Sigma). 10 μ L was then transferred to the counting chamber and counted under a phase contrast microscope. Viable cells remain translucent by excluding Trypan blue, while dead cells are stained blue. Viability was expressed as the proportion of viable cells to the total number of cells.

2.2.8 Cell cryopreservation and thawing

Cells were frozen using a freezing mix containing 40% DMSO (Sigma), 40% foetal calf serum and 20% RPMI 1640 media. Cells were counted and centrifuged at 1200 rpm for 5 minutes to form a cell pellet. The cell pellet was then resuspended in 0.5ml of CM and added to 0.5ml of freezing mix. Cells were transferred into a 1.5ml cryovial (Cryotube Vials, Nunc®, Denmark) and placed in a Nalgene™ Cryo 1°C

freezing container (Fisher Scientific, Loughborough, UK) containing 250ml of 100% isopropyl alcohol (BDH) at -70°C. After 24 hours the cryovials were transferred to liquid nitrogen containers for long term storage. Up to 10×10^6 cells were frozen in a single aliquot. When required, cells were thawed rapidly in a 37°C water bath, diluted in 15mls of CM and washed by centrifugation at 1200 rpm for 5 minutes. The cell pellet was then resuspended in 1ml of CM and counted. Cells were then allowed to recover in CM at 37°C for 24 hours prior to setting-up any assay.

2.2.9 Generation of EBV-transformed B lymphoblastoid cell lines

Previously titrated Epstein-Barr virus from the B95D8 EBV-producing marmoset cell line was added to a dry cell pellet at a concentration of 50µl per 1×10^6 PBMCs and incubated for 1 hour at 37°C, flicking the cells at 15 minute intervals to ensure adequate mixing. The cells were resuspended in CM and 0.5µg/ml of previously titrated phytohaemagglutinin (PHA, Sigma) was added. PBMCs were aliquoted into a 24 well plate at 1×10^6 PBMCs/ml and incubated at 37°C for 3-4 weeks. After this period, the resultant cell population of transformed LCLs was transferred to a culture flask and grown indefinitely or cryopreserved.

2.2.10 Characteristics of EBV-transformed B lymphoblastoid cell lines

Where possible, LCLs were generated from all healthy volunteers and patients used in this study. LCLs from the 10th International Histocompatibility Workshop cell panel (Yang *et al.*, 1989) were also kindly donated by Dr S.G.E Marsh, HLA Informatics group, ANT).

2.2.11 EBV specific T cell stimulation using LCLs or HLA-restricted EBV peptides

EBV specific T cells were stimulated with either HLA restricted EBV peptides or EBV-transformed LCLs at 37°C for 4, 16 or 40 hours (**Figure 2.1**).

1 ml aliquots of red blood cell lysed serum-free whole blood, PBMCs at $1 \times 10^6/\text{ml}$ or lymph node cell suspensions at $1 \times 10^6/\text{ml}$ were added into 24-well plates for peptide stimulation experiments. HLA specific EBV peptides were selected according to the sample's HLA type and added at final concentrations of 0.1, 1 and $10 \mu\text{g}/\text{ml}$. The final concentration of DMSO did not exceed 1%.

For LCL stimulation experiments, 1ml aliquots of autologous EBV-transformed LCLs and LCLs matched for a single or multiple Class I HLA allele were used (**Table 2.3**) at a concentration of $1 \times 10^6/\text{ml}$.

Negative controls were the responder cell populations incubated alone (unstimulated sample), or with EBV peptides not presented by the HLA molecules expressed by the cells (HLA-irrelevant peptides), and PBMCs incubated with at least two HLA-mismatched LCLs. Stimulation with PHA (Sigma) was included as a positive control at a final concentration of $10 \mu\text{g}/\text{ml}$. Prior to incubation, Brefeldin A (Sigma) reconstituted in ethanol was added to each condition at a final concentration of $10 \mu\text{g}/\text{ml}$. Brefeldin A blocks protein transport from the ER to the Golgi apparatus and thereby ensures all cytokines produced by EBV-specific stimulation remain within the cells and can be detected by intracellular cytokine staining (**Section 2.3.2**).

Anti-human CD28 antibody (R&D systems) was also added to the culture at the time of Brefeldin A in one set of experiments using a range of different concentrations.

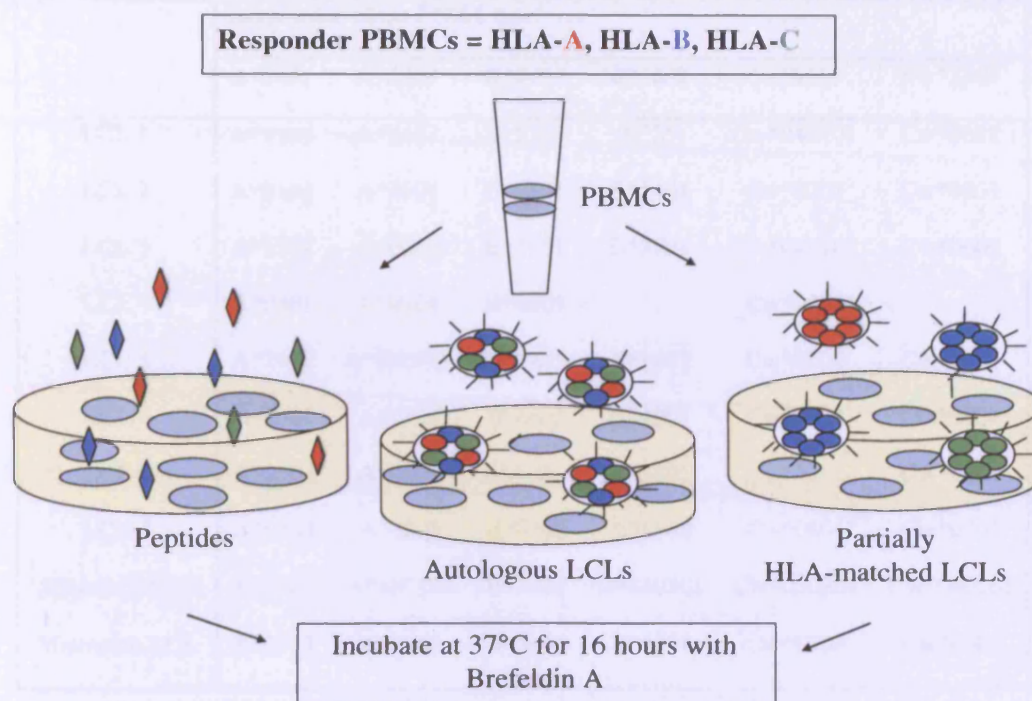
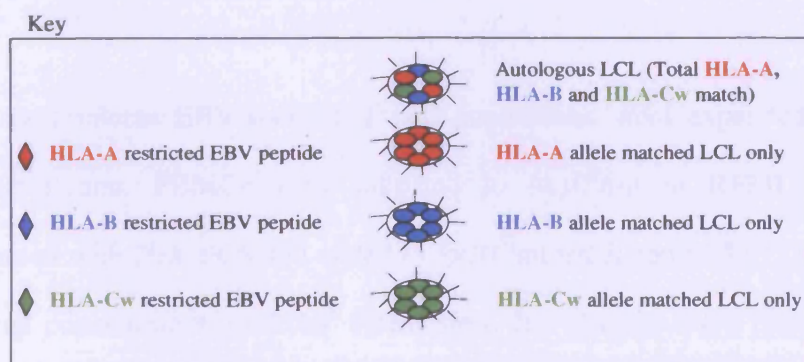


Figure 2.1: Schemata to show method of EBV specific T cell stimulation

PBMCs are stimulated with HLA specific EBV peptides, autologous EBV-transformed LCLs or HLA-matched LCLs at 37C in the presence of Brefeldin A.



	<i>Responder Class I HLA type</i>					
	<i>A*0101</i>	<i>A*0201</i>	<i>B*4402</i>	<i>B*1801</i>	<i>Cw*0501</i>	<i>Cw*1203</i>
LCL 1	A*0101	A*2402	B*5701	B*35	Cw*040101	Cw*0602
LCL 2	A*0101	A*2601	B*0801	B*1401	Cw*0701	Cw*0801
LCL 3	A*1101	A*0201	B*5001	B*3501	Cw*040101	Cw*0602
LCL 4	A*0301	A*0201	B*4001		Cw*0304	
LCL 5	A*2902	A*680102	B*4402	B*1401	Cw*0801	Cw*1601
LCL 6	A*0301	A*1101	B*4402	B*4901	Cw*0801	Cw*0701
LCL 7	A*2501	A*1101	B*35	B*1801	Cw*1203	Cw*0401
LCL 8	A*0301	A*3003	B*0702	B*1801	Cw*0801	Cw*0702
Mismatch LCL 1	A*2601	A*680101	B*3501	B*440301	Cw*030301	Cw*040101
Mismatch LCL 2	A*2301	A*3301	B*5801	B*4901	Cw*0701	Cw*0302

Table 2.3: Example of a panel of partially HLA-matched LCLs used for one healthy individual

The HLA type of the healthy volunteer is shown at the top of this table. 4 pairs of LCLs were selected that match on a single Class I HLA allele only. At least two LCLs that were mismatched on all Class I HLA alleles were selected for negative controls

In some experiments EBV-specific T cell populations were expanded *in vitro* by repeat stimulation. PBMCs were adjusted to 4×10^6 /ml in RPMI 1640 media supplemented with 20% FCS and added to 1×10^5 /ml irradiated LCLs (13,000 rads) to give a final concentration of 2×10^6 PBMCs/ml. 2ml aliquots were plated into a 24-well plate and incubated at 37°C. On day 9, and every 7 days thereafter, cells were removed from culture, spun into a cell pellet, resuspended and adjusted to 1×10^6 /ml using a combination of the preserved supernatant (50%) and fresh RPMI 1640 media containing 20% FCS (50%). Cells were then re-stimulated with freshly irradiated

LCLs at a ratio of 4 lymphocytes: 1 LCL and re-plated in 2ml aliquots into clean 24-well plates.

After each re-stimulation, an aliquot of cells was taken from the culture, incubated for 16 hours in the presence of Brefeldin A, and stained with immunofluorescent antibodies (**Section 2.3.2**). From day 14 IL-2,(R&D Systems) was added at 20IU/ml to the culture every 2-3 days.

2.2.12 Tumour cell lines used for immunohistology

EBV-transformed LCLs were generated *in vitro* and cultured in CM, as described previously. Two human herpes virus 8 (HHV8)-positive, EBV-negative cell lines, BC-3 and BCP-1 were kindly provided by D. Bourboulia (Cancer Research UK Viral Oncology Group, UCL). BC-3 cell line required 20% heat inactivated foetal calf serum in the CM. The cytomegalovirus (CMV)-positive, EBV-negative cell line BJ1 was kindly provided by Dr K. Lawson (Dept of Virology, RFUCMS) and generated by infecting EBV-negative fibroblasts with the AD 169 strain of CMV.

2.2.13 *Mycoplasma* testing and treatment

Cell lines used in this study were tested for contamination by *Mycoplasma* every 4-6 weeks using a PCR based kit (VenorGem®, Minerva Biolabs, Berlin, Germany). Cell lines were harvested, centrifuged into a cell pellet and the supernatant decanted into a 15ml conical tube. The PCR sample was prepared by briefly heating the supernatant (95°C for 5mins) and centrifuging again to remove cell debris. 2µl of supernatant was used as the PCR sample. The reaction volume of the PCR was 50 µl, which contained 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 3.0 mM MgCl₂, 0.05 mM deoxynucleotide triphosphates (dNTPs), 0.2 µM of each primer and 1 unit of a *Thermus aquaticus*

(*Taq*) DNA polymerase (Invitrogen). The PCR reaction was executed by a Peltier thermal Cycler 200, (MJ Research Inc, Massachusetts, US) with an initial cycle of 94°C for 2 minutes, 55°C for 2 minutes, 72°C for 2 minutes, followed by 34 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute. This was followed by a final cycle of 72°C for 4 minutes and 4°C indefinitely. A previously amplified DNA-fragment of *Mycoplasma orale* was used as a positive control, and as a negative control the sample was substituted with nuclease-free water. The amplified PCR product was separated by DNA electrophoresis in a 1.5% agarose gel (Section 2.4.4).

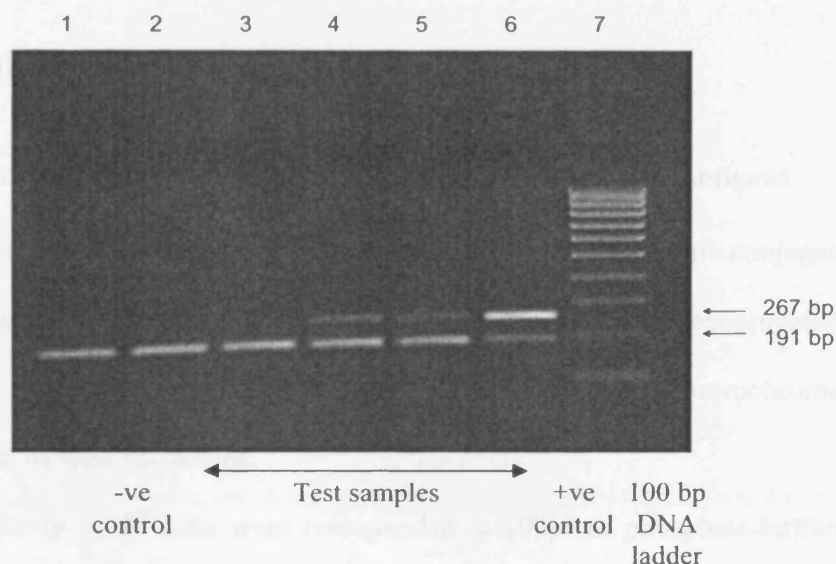


Figure 2.2: Mycoplasma detection

Mycoplasma contamination was detected using a PCR-based kit (VenorGem®, Minerva Biolabs, Berlin, Germany). Positive contamination is illustrated by two bands; one at 267bp representing the *mycoplasma* amplicon and a second at 191bp representing the internal control amplicon. The lane numbers represent the following samples. Lane 1 = negative control. Lane 2 = negative LCL sample. Lane 3 = previously contaminated sample successfully treated with mycoplasma removal agent. Lanes 4/5 = contaminated samples. Lane 6 = positive control. Lane 7 = 100bp DNA ladder.

All samples should possess a band of 191 base pairs (bp) as an internal control to indicate successful DNA amplification. *Mycoplasma* contamination was indicated by an additional band 267 bp (**Figure 2.2**).

Mycoplasma contamination was treated by adding 0.5µg/ml of mycoplasma removal agent (MRA, MP Biomedicals, London, UK) to the infected cell line in CM and incubating for 1 week. Cells were then transferred to CM without additional MRA and grown for a further week. Successful removal of *mycoplasma* contamination was confirmed by the VenorGem® PCR detection kit.

2.3 Cellular assays

2.3.1 Immunofluorescent antibody staining for cell surface antigens

Cells were either stained directly using specific antibodies that were conjugated with a fluorochrome, or indirectly with a two-stage technique, using unconjugated primary antibodies (mouse IgG or mouse IgM), followed by secondary fluorochrome-labelled anti-mouse isotype antibodies.

Approximately 1×10^5 cells were resuspended in 100µl of phosphate-buffered saline (PBS, Oxoid, Basingstoke, UK) with 0.2% sodium azide and 0.2% bovine serum albumin (BSA) in a 96-well U bottomed plate. Saturating concentrations of antibodies were added to the cells and incubated at 20°C for 15-30 minutes. The staining plate was then centrifuged at 1200 rpm for 5 minutes, flicked to remove the supernatant and washed in 150µl PBS with 0.2% sodium azide and 0.2% BSA. Where an indirect staining method was used to characterise cell surface activation markers, this washing step was repeated four times to remove excess primary antibody prior to adding the

secondary antibody. 10 μ l of Fluorescein isothiocyanate (FITC) conjugated (Fab2) goat anti mouse IgG and Phycoerythrin (PE) conjugated goat anti mouse IgM was added to the cells and incubated at 20°C for 15-30 minutes. The cells were then washed and fixed by resuspending in 50 μ l 1% paraformaldehyde diluted in PBS to prevent dissociation of antibody from their ligands. Fixed cells were kept in the dark at 4°C and analysed by flow cytometry (**Section 2.3.4**) within 7 days.

2.3.2 Immunofluorescent antibody staining for intracellular antigens

Following EBV-specific T cell stimulation using EBV peptides or LCLs in the presence of Brefeldin A, cells were removed from the 24-well plate, transferred to 15ml conical tubes (BD), washed in PBS with 0.2% sodium azide and 0.2% BSA and centrifuged into a cell pellet. The supernatant was discarded and the cell pellets were resuspended in 150 μ l of PBS with 0.2% sodium azide and 0.2% BSA, and transferred to a 96-well plate. Cells were centrifuged and washed as before and resuspended in 100 μ l of the fixation medium, Reagent A (Caltag Laboratories). The cells were incubated at room temperature for 12 minutes in the dark. After incubation, the cells were centrifuged and washed as before in 150 μ l of PBS with 0.2% sodium azide and 0.2% BSA and resuspended in 100 μ l of permeabilization medium, Reagent B (Caltag). Cells were then incubated in the dark at room temperature for an additional 12 minutes. Cells were again washed as above and stained with saturating concentrations of previously titrated anti-CD3 Cy5 (Caltag), anti-CD8 FITC and anti-IFN γ PE (Pharmingen, BD) (**Section 2.3.2**).

2.3.3 Immunofluorescent antibodies

All antibodies used in this study are listed in **Tables 2.4 and 2.5**. Directly conjugated antibodies were purchased from BD Pharmingen, San Diego, USA or Caltag, Buckingham, UK and titrated prior to use. Unlabelled antibodies used in the indirect method of staining were generated from in-house hybridomas or purchased from suppliers as indicated in **Tables 2.4 and 2.5**.

<i>Name</i>	<i>Fluorochrome</i>	<i>Source</i>	<i>Target and effects</i>
CD3	Cy5	Caltag	T cell receptor
CD8	FITC	BD Pharmingen	T cell co-receptor
CD86	FITC	BD Pharmingen	B7.2 ligand. Binds to CD28 during cell activation
CD83	PE	BD Pharmingen	Costimulatory ligand
HLA-DR	PE/FITC	BD Pharmingen	MHC class II cell surface molecule
IFN- γ	PE	BD Pharmingen	Produced by T-helper cells, CD8 ⁺ cells NK cells. Activates macrophages, increases MHC-class I and II molecules, increases antigen presentation
TNF- α	PE	BD Pharmingen	Produced by CD8 ⁺ cells
IL-2	PE	BD Pharmingen	Produced by T cells. Promotes T-cell, B-cell and NK-cell proliferation
IL-4	PE	BD Pharmingen	Produced by mast cells and T _H 2 cells. Promotes T _H 2 differentiation.
IL-10	PE	BD Pharmingen	Produced by T _H 2 cells. Promotes T _H 2 differentiation.

Table 2.4: Directly conjugated fluorochrome- labelled antibodies used in this study

<i>Name/CD No</i>	<i>Clone</i>	<i>Source</i>	<i>Reactivity</i>
CD19	APC5	SBA	Early B cell lineage, Follicular dendritic cells
	RFB9	Dept. Immunology , RFUCMS	
CD3	OKT3	American Type Cell Collection (ATCC)	Thymocytes, T cells, NK1-T cells
	TCR $\gamma\delta$	Tissue culture services	T cell receptor $\gamma\delta$ heterodimer
CD25	RFT5 γ	Dept. Immunology , RFUCMS	α subunit of IL-2 cytokine receptor
CD26		Pharmingen	Co-stimulatory molecule in T cell activation
CD69	AIM	Biogenesis	Early activation marker for activated leukocytes
	RFDR2	Dept. Immunology , RFUCMS	MHC class II
CD38	RFT10	Dept. Immunology , RFUCMS	Activated T and B cells, plasma cells, germinal centre B cells
CD45R _A	SN130	Dept. Immunology , RFUCMS	Naïve T cells
CD45R _O	UCLH1	UCL, P. Beverley	Effector T cells
CD4	RFT4	Dept. Immunology , RFUCMS	MHC class II-restricted cells, some thymocytes, monocytes, macrophages, granulocytes
CD28	9.3 IgG	M. Glennie, Southampton	T cells, plasma cells.
CD7	RFT2	Dept. Immunology , RFUCMS	T cells, Thymocytes, NK cells
CDw150	SLAM	A.Immunochemical	Thymocytes, Subset of T and B cells. Involved in B cell co-stimulation

Table 2.5: Antibodies used in the indirect method of staining for the characterisation of cell surface activation markers

2.3.4 Multi-parameter flow cytometry

Detection of immunofluorescent antibody labelled cells was performed using a Calibur (BD) Fluorescent Activated Cells sorter (FACs) flow cytometry system. This instrument is able to analyse size, granularity and fluorescence of single intact cells in suspension using dual lasers and four different detectors. When a cell passes the laser beam, light is deflected from the detector, and this interruption of signal from the laser is recorded. Cells with a fluorescently tagged antibody attached to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system at right angles to the laser beam. The argon-ion laser of the FACsCalibur has an emission peak of 488nm and is able to detect size (Forward Scatter, FSC), granularity (Side Scatter SSC) and the fluorochromes FITC, PE, Cyanine 5 (Cy5) and Peridinin chlorophyll protein (PerP). The second red-diode laser has an emission peak of 635nm and is able to detect the fluorochrome Allophycocyanin (APC). Acquisition of fluorochrome labelled cells was performed using the Cellquest™ software version 3.3 (BD) and subsequently analysed using the FlowJo software (Treestar). Wherever possible, data was collected from 200,000 live cells in the lymphocyte gate for each sample.

2.3.5 Statistical analyses

Prism® 4 Software (Graphpad, San Diego, USA) was used in this thesis to calculate the mean totals and the standard deviation (sd). Data sets were compared using either the non-parametric Mann Whitney U test or unpaired t tests. Statistical correlation between data was also performed using Pearson's rank correlation coefficient.

2.4 Molecular Biology

2.4.1 RNA extraction

Total RNA was extracted from PBMCs that had been stimulated by EBV peptides or LCLs in culture, using the RNeasy Mini Kit™ (Qiagen, UK) according to the manufacturer's instructions. Throughout the procedure, RNA degradation was avoided by using RNase-free plastic ware, washing all pipettes and surfaces with RNaseZap® and molecular grade water (Sigma, Aldrich, St Louis, USA), and changing gloves regularly.

Cells were harvested, washed in ice-cold PBS and centrifuged for 5 minutes at 13,000 rpm. The supernatant was completely removed from the cell pellet and cells were lysed by adding 350µl of RLT Buffer (Lysis buffer, Qiagen) with 1% β-Mercaptoethanol (β-ME). Genomic DNA within the cell lysates was sheared by passing the sample at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. RNA extraction was either performed immediately or the cell lysates were frozen at -80°C and then thawed at 37°C for 20 minutes prior to use.

350µl of 70% molecular grade ethanol was added to the cell lysate and transferred to an RNeasy mini column placed inside a collection tube. The ethanol ensured appropriate conditions for the total RNA to bind to the silica-gel membrane. The columns were centrifuged for 15 seconds at 10,000 rpm and the flow-through discarded. The samples were then washed in 350µl of RW1 buffer and treated with an on-column DNase digestion kit (Qiagen) to remove residual genomic DNA. 10µl of DNase I stock solution was diluted in 70µl of RDD buffer mixed gently, pipetted directly onto the silica-gel membrane and incubated at room temperature for 15

minutes. After incubation the DNase was removed with 350µl of RW1 buffer, followed by two further washes with ethanol-containing RPE buffer. After each wash the columns were centrifuged for 15 seconds at 10,000 rpm and the flow-through discarded. To avoid any carryover of RPE Buffer, the column was transferred to a new collection tube and centrifuged at 10,000 rpm for 2 minutes. RNA was eluted from the membrane and collected into a sterile RNase-free collection tube by adding 50µl of RNase-free water to the column and centrifuging the column at 10,000 rpm for 1 minute. When the expected RNA yield was low (<30µg), a second elution was performed by pipetting the first eluate back onto the silica-gel membrane and repeating the centrifugation. RNA was used immediately as a template for complementary DNA (cDNA) synthesis or stored at -80°C until required.

2.4.2 Generation of complementary DNA

cDNA was generated by reverse transcribing total RNA using Murine Moloney Leukaemia Virus reverse transcriptase (M-MLV RT, Promega, Southampton).

Approximately 1µl of RNA was mixed with 0.5µg random primers (Promega, 500µg/ml) and 1µl of 10mM dNTPs in an RNase-free microcentrifugation tube. In order to remove secondary structures, the RNA, random primers and dNTPs were heated to 70°C for 5 minutes in a hot-block (Block Thermostat, Grant Instruments Ltd, Cambridge UK) and then cooled immediately on ice for a further five minutes to prevent secondary structures reforming. Each sample was centrifuged for 15 seconds at 10,000 rpm and then added to 8µl of master mix (detailed in **Table 2.6**) and mixed well. To check that all genomic DNA had been degraded by the on-column DNase digestion step included in the RNA extraction procedure, a duplicate tube was made

up for each RNA sample as a negative control. These tubes contained all the reagents except for the M-MLV RT which was replaced with nuclease-free water (-RT sample). Tubes were then incubated in a hot-block at 37°C for 60 minutes followed by 70°C for 10 minutes to inactivate the M-MLV RT enzyme. cDNA samples were either kept on ice and used immediately in PCR reactions (Section 2.4.3 and 2.4.4) or stored at -20°C until required.

<i>Reagent</i>	<i>Volume (x1 reaction)</i>
5x M-MLV buffer (Promega)	4µl
Dithiothreitol (DTT) 0.1M (Promega)	2µl
RNase inhibitor 40U/µl (RNaseIn, Promega)	1µl
M-MLV RT 200U/µl (Promega) (+RT samples) OR nuclease-free water (-RT samples)	1µl
Total volume	8µl

Table 2.6: Reagents used in the synthesis of cDNA from RNA

This table lists the reagents used in the master mix for generating cDNA from total RNA in the reverse transcriptase reaction. The test samples contain M-MLV RT and are labelled +RT samples. To check genomic DNA is not present in the samples, a duplicate tube is made up for each RNA sample where the M-MLV RT is replaced with nuclease-free water (-RT sample).

2.4.3 Gene amplification by Polymerase chain reaction

Production of cDNA and absence of genomic DNA in the RNA preparation was verified by amplification of the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene by conventional PCR.

GAPDH was amplified from the DNA template using 5' and 3' primers (**Appendix 1.4**), dNTPs, the heat stable DNA polymerase, *Taq* and buffer containing magnesium. A reaction master mix was prepared according to **Table 2.7**, and added to 2 μ l of +RT and -RT samples. A sample in which cDNA was replaced with nuclease-free water and a sample containing a previously amplified GAPDH PCR product diluted 1:1000-1:5000, were used as negative and positive controls respectively.

PCR reactions were run on a Peltier Thermal Cycler-200 (MJ Research, Inc, Massachusetts, US) according to the protocol in **Table 2.7**.

<i>Reagent</i>	<i>Volume</i> (μ l)	<i>Temperature</i> ($^{\circ}$ C)	<i>Time</i> (mins)	<i>Purpose</i>	
10x <i>Taq</i> buffer with Mg ⁺⁺ (Promega)	5	95	5	Denaturation	
5' primer 100 μ M (Sigma)	0.2	36 cycles {	94	1	Denaturation
3' primer 100 μ M (Sigma)	0.2		55	1	Annealing
dNTPs 10mM (Biogene)	1.0		72	1	Extension
<i>Taq</i> 5U/ μ l (Promega)	0.5	72	10	Extension	
Nuclease-free water	41.1	4	forever	storage	
cDNA template	2				
Total volume	50				

Table 2.7: Reagents and the PCR thermal cycler program used to amplify GAPDH by conventional PCR

2.4.4 DNA electrophoresis

Successful amplification of the target gene was confirmed by electrophoretic separation of the PCR product on an agarose gel. A 2% agarose gel was prepared by

dissolving 4g of electrophoresis grade agarose (Invitrogen) in 200ml of Tris-boric acid-EDTA buffer (TBE: 0.1M Tris, 0.09M boric acid, 1mM EDTA, Biowhittaker). Ethidium bromide (1µg/ml final concentration, Sigma) was added to the gel to visualise DNA. The gel was poured into a sealed gel tray (Biorad, Hercules, California, US) with combs to create loading wells, and left to solidify. Once set, the combs were removed and the gel was covered with TBE buffer. 20µl of PCR product was added to 1µl of Orange G loading buffer (0.1mg final concentration, Pel Freez, Merseyside, UK) and pipetted into the loading chambers. A DNA ladder (DNA Hyperladder IV, Bioline, London) was added to the first and last lanes of each gel. Electrophoresis was performed by running the gel for 30-60 minutes at 130 volts. Once adequate separation of the DNA bands was achieved, the gel was removed and photographed under UV light using molecular analyst® software (Biorad, Hercules, California, US)

An example of a DNA electrophoresis agarose gel showing amplification of GAPDH is shown in **Figure 2.3**.

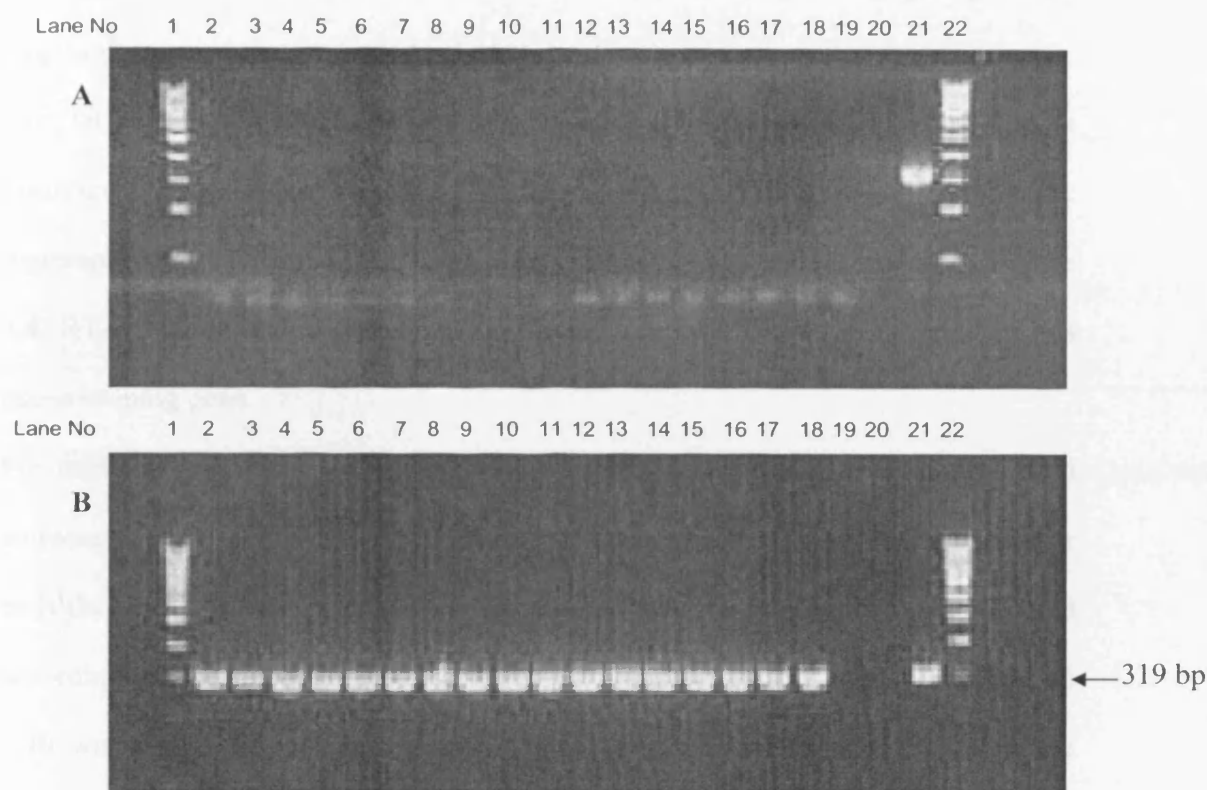


Figure 2.3: A representative example of GAPDH amplification by conventional PCR

Successful production of cDNA and amplification of GAPDH was verified using conventional PCR. Positive amplification of GAPDH is represented by a band at 319 bp. Lanes 1+22 = DNA bp ladder. Lanes 2-18 = test samples. Lane 19 = negative control. Lane 21 = positive control. **Panel A** represents samples that had no reverse transcriptase added and show no GAPDH amplification and **Panel B** represents samples that had reverse transcriptase and show successful GAPDH amplification in all test samples.

2.4.5 Quantitative real-time PCR (RT-PCR)

Quantitative real-time RT-PCR was performed throughout this work using an ABI Prism[®] 7500 (Applied Biosystems, Foster City, California, US). This technique uses fluorescence emitted during the exponential phase of the PCR reaction as an indicator of amplicon production during each PCR cycle, and thereby allows accurate

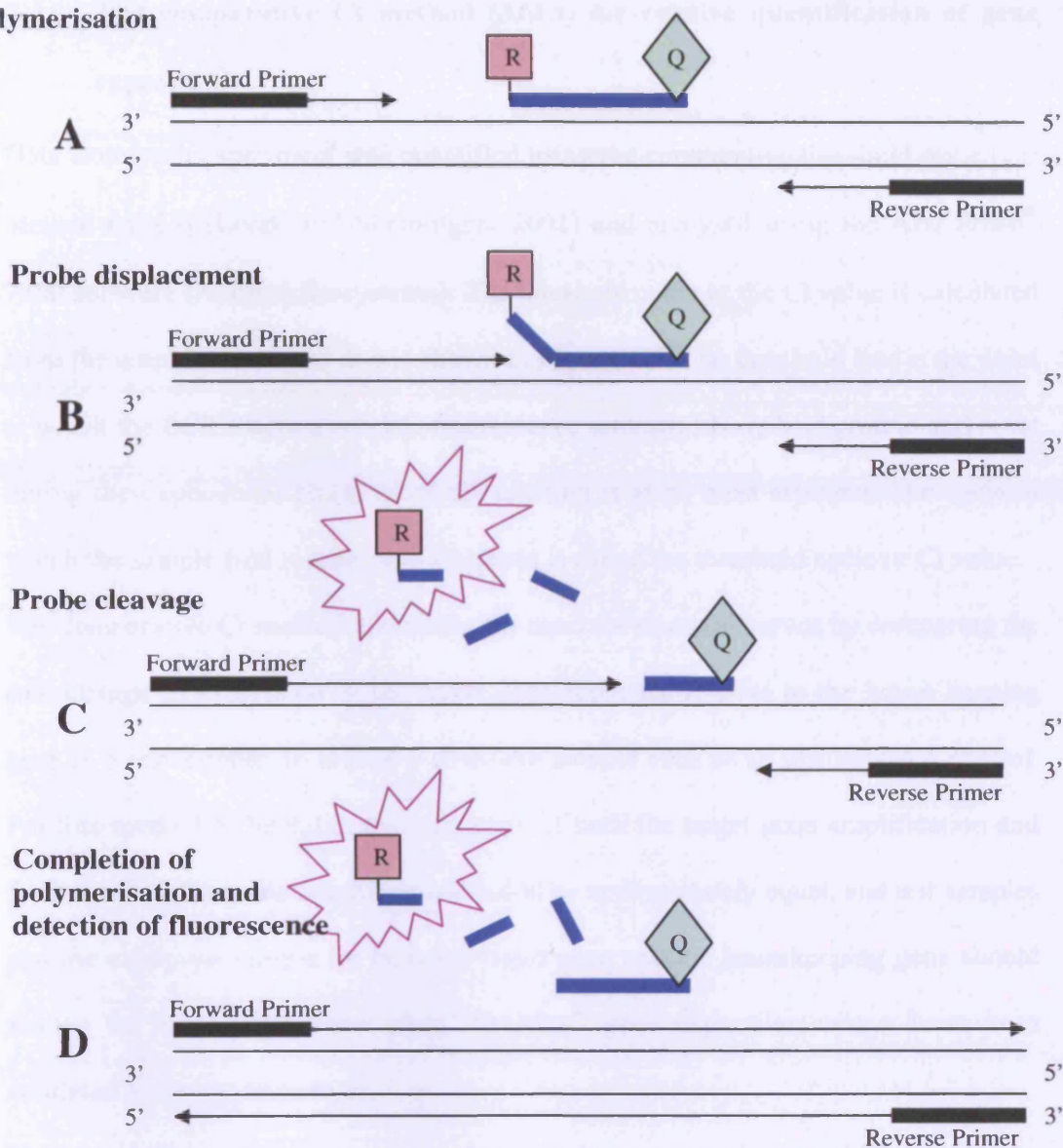
quantitation of RNA. Gene expression was determined by using pre-optimised TaqMan[®] gene expression kits (Applied Biosystems).

Each kit includes a forward and a reverse primer to amplify the gene of interest, and a fluorescent labelled internal probe specific for the amplified gene. A schematic representation of the amplification and detection of fluorescence is shown in **Figure 2.4**. RT-PCR reactions were performed for the gene of interest and an endogenous house keeping gene.

For each experiment, a reaction plate was designed using the ABI Prism[®] 7500 software (Applied Biosystems). Each cDNA sample was run in triplicate wells for both the gene of interest and the housekeeping gene. The mastermix was prepared according to **Table 2.8** and dispensed into the reaction plate. 1 μ l of cDNA, diluted 1:10 with nuclease-free water was added to each well and run according to the thermal cycler conditions listed in **Table 2.8**.

<i>Reagent</i>	<i>Volume (μl)</i>	<i>Temperature ($^{\circ}$C)</i>	<i>Time (mins)</i>	<i>Purpose</i>
2x TaqMan [®] universal Master Mix (Applied Biosystems)	12.5	95	10	Denaturation
TaqMan [®] gene expression assay (Applied Biosystems)	1.25			
Nuclease-free water	10.25			
		40 cycles { 95 60	15 secs 1	Denaturation Annealing and extension
Total volume	24			

Table 2.8: Protocol for quantitative RT-PCR using the TaqMan[®] gene expression kit

Polymerisation**Figure 2.4: The TaqMan® gene expression assay.**

After denaturation, two template-specific primers anneal to the cDNA copy of the mRNA defining the ends of the amplicon. A third oligonucleotide probe (■) then hybridizes to the amplicon (A). This hydrolysis probe is conjugated with a non-fluorescent quencher (Q) dye at the 3' end which absorbs the fluorescence of the reporter (R) FAM dye at the 5' end, as long as the probe remains intact. Polymerization proceeds at the same temperature as the annealing step causing displacement and hydrolysis of the labeled probe (B). The reporter dye is cleaved by the 5'-nuclease activity of *Taq* (C) and released from the close proximity of the quencher dye. Fluorescence is detected and polymerization is completed (D). During each consecutive PCR cycle the fluorescence increases due to the progressive and exponential accumulation of free reporter fluorochromes.

2.4.6 The comparative Ct method ($\Delta\Delta C_t$) for relative quantification of gene expression

Data from each experiment was quantified using the comparative threshold cycle (C_t) method ($\Delta\Delta C_t$) (Livak and Schmittgen, 2001) and analysed using the ABI Prism[®] 7500 software (Applied Biosystems). The threshold cycle or the C_t value is calculated from the amplification plot and is shown in **Figure 2.5**. The threshold line is the point at which the PCR reaction reaches fluorescence intensity above background and is set during the exponential phase when the reaction is at its most efficient. The cycle at which the sample first reaches this threshold is called the threshold cycle or C_t value.

The comparative C_t method eliminates the need for standard curves by comparing the fold change in expression of the target gene sequence relative to the house keeping gene in a test sample, to that of a calibrator sample such as an unstimulated control. For this method to be valid, the efficiency of both the target gene amplification and the house keeping gene amplification should be approximately equal, and test samples and the calibrator sample for both the target gene and the housekeeping gene should always be run on the same plate. TaqMan[®] gene expression assays have been validated by the manufacturer.

The calculations used to quantitate the data from a sample spreadsheet using the comparative C_t method are shown in **Figure 2.5**.

Well	Details	House-keeping gene Ct value	Target gene Ct value	ΔCt (Target gene Ct - House-keeping gene Ct)	Mean ΔCt for calibrator sample	$\Delta\Delta Ct$ (ΔCt test sample - Mean ΔCt calibrator sample)	$2^{-\Delta\Delta Ct}$
a1	Calibrator sample	26.718	36.569	9.851	9.819		
a2		26.444	36.12	9.676			
a3		26.553	36.484	9.931			
b1	Test Sample	26.632	32.337	5.705		-4.114	17.704
b2		26.616	32.483	5.867		-3.952	15.824
b3		26.761	32.613	5.852		-3.967	16

Figure 2.5: A representative spreadsheet of data showing calculations used in the comparative threshold Ct method

The samples were analysed using quantitative RT-PCR and Ct values were obtained. The mean fold change in expression of target gene was normalised using the $\Delta\Delta Ct$ method, where $\Delta Ct = Ct$ (target gene) - Ct (house-keeping gene), and $\Delta\Delta Ct = \Delta Ct$ (test sample) - ΔCt (calibrator). Gene expression was then converted into arbitrary normalized units using the equation $2^{-\Delta\Delta Ct}$.

2.5 Immunohistology

2.5.1 Preparation of Cytospins

150 μ l of 1×10^6 cells/ml resuspended in PBS was spun onto slides coated with poly-L-lysine (PLL) or 3-aminopropyltriethoxysilane (APES) using cytocentrifuge carriers. The slides were removed immediately after cytocentrifugation and dried overnight at room temperature. The following day the slides were fixed with fresh acetone and frozen at -40°C wrapped in aluminium foil.

2.5.2 Preparation of fresh tissue sections

A piece of tissue 3-5mm² was cut using a scalpel and mounted onto a cork base with tissue mounting gel (Tissue-Tek® O.C.T. Compound). The block was then frozen in iso-pentane which had been cooled in liquid nitrogen. Once frozen the blocks were removed and stored at -70°C. Six micron sections were cut from the block using a Frigocut Cryostat and placed onto PLL slides. The sections were left to dry overnight and then fixed in acetone (BDH, AnalaR quality) for 10 minutes followed by absolute alcohol (Hayman Ltd, Waitham, UK, AnalaR quality) for a further 10 minutes. Once dry, the tissue sections were stored at -40°C, until required for staining.

2.5.3 Preparation of formalin-fixed tissue sections

Paraffin-embedded sections that had been prepared according to standard histological techniques were cut onto sialin-coated slides and paraffin removed by one of two methods. Sections were either placed in xylene and 100% ethanol and microwaved in 10mmol/L citrate buffer, pH 6.0, at 600W for 5, 10, 15 and 20 minutes, (Shi *et al.*, 1991) or incubated at 37°C with Tris buffed saline, pH 7.8, containing 0.05% trypsin, 0.05% chymotrypsin and 0.1% calcium chloride for various times.

2.5.4 Antibodies used to detect EBV antigen

The antibodies used in the detection of EBV by immunohistochemistry are listed in **Table 2.9**. The murine LN53 antibody was kindly provided by Professor C Boschoff, Centre for Virology, RFUCMS (Dupin *et al.*, 1999). Normal mouse serum (Harlan Sera-lab) was used as a negative control at a dilution of 1/2000 during immunoperoxidase staining.

The RFD3 antibody was recloned from the hybridoma RFD3 line produced by the Clinical Immunology department, RFUCMS, prior to staining to ensure specificity.

<i>Name</i>	<i>Clone</i>	<i>Class/Subclass</i>	<i>Source</i>	<i>Reactivity</i>
Murine anti-LMP 1	CS 1-4	IgG ₁ kappa	Dako	LMP 1, EBV
Murine anti-LMP 2	CS 1-4	IgG ₁ kappa	Dako	LMP 2, EBV
Murine anti-EBNA 2	PE2	IgG ₁ kappa	Dako	EBNA 2, EBV
Murine LN53	LN53		Centre for Virology, RFUCMS	LNA 1, HHV8
Murine RFD3	RFD3	IgG ₂	Clinical Immunology, RFUCMS	EBV

Table 2.9: Antibodies used to detect EBV by immunoperoxidase staining

2.5.5 Detection of EBV by immunoperoxidase staining

Slides prepared from cytopins or fresh tissue sections were removed from -40°C and allowed to reach room temperature. 50µl of PBS containing 10% normal human serum (used to block non-specific binding of the antibody via the Fc receptor) and 10% aprotinin (used to block proteolytic enzymes) was added to each section. Previously titrated primary antibodies were then added to the sections and incubated for 1 hour at room temperature. This was followed by incubation with a second layer of Peroxidase-conjugated rabbit anti-mouse Ig (Code no P0260, Dako, Ely, Cambs) for a further hour at room temperature. After each incubation, sections were then washed in freshly prepared PBS for 5 minutes, fixed in formalin buffered saline, pH

7.6 for 10 minutes, and washed again in PBS. Results from the staining were visualised by incubating the sections in DAB Peroxidase Substrate Solution (0.05% DAB, (Sigma) 0.05% Nickel Ammonium Sulfate and 0.015% H₂O₂ in PBS, pH7.2) for 5 minutes. The slides were then washed in PBS, stained in cobalt chloride solution for 5 minutes, rinsed in distilled water for 5-10 seconds and counterstained for 2 minutes with Nuclear Fast Red solution (Sigma). Finally, sections were washed in running tap water for 5 minutes and dehydrated by progressive immersions in 70%, 90% and 100% alcohol, followed by 50% alcohol/50% citoclear (HD supplies, Aylesbury, Bucks) and 100% citoclear.

2.5.6 Detection of EBV by complement indirect immunofluorescent technique

Sections prepared from cytopins or fresh tissue sections were initially rinsed in freshly prepared complement fixation buffer (CFB, Oxoid) and incubated with heat inactivated human immune serum and human complement, diluted in CFB for 1 hour at 37°C. Human immune serum was taken from a healthy EBV sero-positive donor as a source of anti-EBNA antibodies. Human immune serum from a healthy EBV sero-negative donor was used as a negative control. Human complement was obtained fresh from EBV sero-negative donors and stored at -70°C. After incubation, the sections were washed twice in CFB and incubated for 45 minutes at room temperature with FITC-conjugated rabbit anti-human complement (Dako). Sections were further washed in CFB and mounted in AF1 citifluor (Citifluor Ltd, London).

2.5.7 Detection of Latent EBV infection by *in situ* hybridisation

Epstein-Barr virus encoded RNA (EBER) was detected by a peptide nucleic acid (PNA) *in situ* hybridization (ISH) Detection Kit (Dako), using a fluorescein-conjugated EBER PNA probe and according to the manufacturer's instructions.

Formalin-fixed, paraffin-embedded tissue sections were de-paraffinised by immersion in xylene and rehydrated by progressive immersions in 99% and 96% ethanol, followed by distilled water. Sections were then pre-treated with 150µl proteins K, diluted 1:10 in Tris-Buffered Saline (TBS), pH 7.5 and incubated for 20-30 minutes. Sections were washed in distilled water and dehydrated in 96% ethanol. Hybridisation was performed by adding 2-3 drops of the fluorescein-conjugated EBER PNA probe and incubating in a humidity chamber at 55°C for 1½ hours. After incubation, the sections were washed in a Stringent Wash solution (PNA ISH Detection Kit, Dako) that had been warmed to 55°C for 25 minutes. 2-3 drops of alkaline phosphatase-conjugated rabbit F(ab) anti-FITC were then added to each section, incubated at room temperature for 30 minutes and washed off in TBS followed by distilled water. Use of the antibody F(ab) fragment ensured that non-specific binding was kept to a minimum. Enzyme substrate, 5-bromo-4-chloro-3-indolylphosphate (BCIP) nitroblue tetrazolium (NBT), combined with an inhibitor of endogenous alkaline phosphatase, levamisole was added to each section and incubated for 30-60 minutes at room temperature. Finally the sections were washed in tap water, counterstained in Nuclear Fast Red solution and mounted.

Chapter 3

**Development of a rapid immuno-assay to enumerate
EBV-specific T cell responses: Healthy Individuals.**

3.1 INTRODUCTION

An effective immune response to EBV is of crucial importance in controlling EBV driven B cell proliferation, as evidenced by the increased frequency and severity of EBV driven malignancies in immunocompromised individuals.

The immune response to EBV has been well characterised. The humoral response to the virus has been quantified in both primary and persistent EBV infection. However, its main effect is in removing circulating virus from the blood with IgG antibodies directed at both lytic and latent EBV antigens and plays no role in controlling EBV-driven B cell proliferation. In contrast, EBV is known to stimulate a vigorous cell-mediated immune response and evidence suggests that this is the main immune mechanism protecting individuals against the development of EBV driven malignancies (Hislop *et al.*, 2001; Rickinson and Moss, 1997; Callan *et al.*, 1998b).

From these and other studies, numerous techniques have been developed to measure the EBV-specific T cell response (**Table 3.1**). Although these techniques were originally designed in the context of research, many have potential for use as a clinical assay. Measuring EBV-specific T cell responses is particularly important in patients with EBV-driven malignancies where manipulation of the immune response may be used to treat the disease. An assay that rapidly and reliably enumerated functional EBV-specific T cells would therefore have widespread clinical application.

An example of a clinical situation where measurement of functional EBV-specific T cell responses would be useful is in patients with PTLN. Withdrawal of immunosuppressive therapy has been shown to lead to regression of PTLN by allowing recovery of anti-EBV immunity so that an increase in the numbers of

functional EBV-specific T cells controls the EBV-driven proliferation (Starzl *et al.*, 1984). However, without an effective assay to measure the recovery of a functional EBV-specific T cell response, reduction in immunosuppression is arbitrary and reliant on the judgment of the clinician responsible for the patient. This therefore increases the risk of graft rejection significantly. An effective assay that enumerates the EBV-specific cellular immune response would therefore enable the clinician to tailor step-wise reductions in immunosuppression to the recovery of EBV-specific immunity. EBV-driven B cell proliferation has the potential for exponential growth, therefore the assay must provide a rapid report to clinicians in order to be clinically effective.

Current techniques used to monitor EBV immunity during reduction of immunosuppression include immunophenotyping for an increase in T cell surface activation markers, particularly MHC class II expression on CD8⁺ T cells (Amlot *et al.*, 1996), indicative of emergence of immune reactivity. Increased frequencies of CD8⁺MHC class II⁺ T cells were originally observed in primary EBV infection (Crawford *et al.*, 1981) and subsequently reported in a patient with PTLD (Leaver *et al.*, 2004; Rees *et al.*, 1998). MHC class II expression has also been shown to correlate with the frequency of viral-specific T cells identified by HLA class I tetramers (Webster *et al.*, 2000), but importantly not with alloreactive T cells in renal transplantation. This non-specific method of monitoring the recovery of cellular immunity as immunosuppression is reduced has been successfully employed in PTLD patients, resulting in a marked reduction in the frequency of graft rejection (Amlot, 2001; Amlot *et al.*, 2007). This method does not require *in vitro* culture and is very rapid. Results can therefore be reported back to the clinician within 2-4 hours of receiving a blood sample. The disadvantage, however, is that it does not provide any

information about the antigen-specificity of the functional T cells and therefore an increase in CD8⁺MHC class II⁺ T cells could be a response to a co-existing viral infection.

Screening for a decrease in EBV viral load is also used to monitor recovery of EBV-specific immunity in patients with PTLD. However, this is often misleading with increased EBV load post transplant not always progressing into PTLD and falls in viral load post diagnosis not always correlating with clinical response. One example of this, as discussed in the Introductory chapter, is use of the anti-CD20 monoclonal antibody, rituximab, which can cause rapid disappearance of detectable EBV genome copy numbers without necessarily leading to effective treatment of the PTLD. A new approach is therefore needed that provides information on EBV antigen specificity as well as the size and functionality of the response.

A variety of assays are used to evaluate EBV-specific immune responses. Each has their own advantages and limitations for use in the context of monitoring treatment for PTLD (**Table 3.1**). Traditionally LDA is the ‘gold standard’ assay and has been used to measure the frequencies of functional EBV-specific T cells in both EBV seropositive individuals (Tan *et al.*, 1999; Kuzushima *et al.*, 1999) and PTLD patients receiving allogeneic CTL infusions (Haque *et al.*, 2001). However, although this assay quantifies the number and function of the cells, the method is laborious requiring several weeks of *in vitro* expansion rendering it unsuitable for clinical use.

The ⁵¹Cr-release assay (Brunner *et al.*, 1968) is also used to detect functional cytolytic EBV-specific CD8⁺ T cells (Hislop *et al.*, 2001; Kuzushima *et al.*, 1999; Crotzer *et al.*, 2000). However, regulatory requirements associated with use of hazardous

radioactive materials limits utility of this assay to laboratories with the necessary accreditation.

More recently fluorochrome-labelled HLA-peptide tetramers have been used to detect and quantify antigen-specific T cells. Originally described in 1996 (Altman *et al.*, 1996), HLA tetramers comprise of 4 biotinylated molecules of a defined HLA:peptide combination bound to fluorescently labelled streptavidin. Multivalency enables sufficient binding affinity to the low affinity T cell receptors specific for the HLA:peptide complex in order that tetramer-bound T cells can be detected by conventional flow cytometry. HLA class I tetramers, have been extensively used to quantify the CD8⁺ T cell response to viral infections such as HIV (Wilson *et al.*, 2000), Hepatitis virus C (HCV (Lechner *et al.*, 2000), CMV (Lacey *et al.*, 2002; Gillespie *et al.*, 2000) and EBV (Tan *et al.*, 1999; Callan *et al.*, 1998b), as well as tumours such as melanoma (Lee *et al.*, 1999; Anichini *et al.*, 1999) and chronic myeloid leukaemia (CML) (Molldrem *et al.*, 2000). HLA tetramer staining alone, however, provides no information as to the functionality of the antigen-specific T cells. This is particularly important in the context of PTLD arising in solid organ transplant patients where EBV-specific CD8⁺ T cells may be present, but not functional, due to immunosuppression. Validity of this concern is supported by studies with EBV seropositive healthy individuals which demonstrated that the frequencies of EBV-specific CD8⁺ T cells obtained by HLA-peptide tetramer staining were significantly higher than those obtained by limiting dilution analysis and ELISpot; a method that assesses functionality (Tan *et al.*, 1999). Tetramers can be combined with intracellular cytokine staining to assess functionality after antigen stimulation, but this adds to the complexity and cost of the assay. A disadvantage with

HLA tetramers is that prior knowledge of the precise antigenic peptide epitope is required. As EBV epitopes have only been identified for common HLA class I alleles, many patients with rare HLA alleles would not be able to be tested using this method. In addition, as tetramers only measure the frequency of T cells specific for one peptide several different tetramers would need to be tested per patient requiring availability of a large spectrum of HLA tetramers to perform a comprehensive assessment of EBV T cell immunity. Another limitation of tetramers is that class II: peptide reagents are not routinely available for detection of CD4⁺ T cells.

A technique that is often used to quantify functional EBV-specific T cells is ELISpot (Bollard *et al.*, 2004; Comoli *et al.*, 2005; Straathof *et al.*, 2005; Tan *et al.*, 1999). This method uses antibodies specific for mediators of T cell function (anti-cytokine, anti-granzyme, or anti-perforin antibodies) that are bound to the surface of a tissue culture plate and capture secreted functional mediators from antigen stimulated T cells. Individual functional T cells are revealed as spots, thereby allowing quantification of antigen-specific T cells. Although this technique is very sensitive, with a threshold detection rate of 20 spots per million PBMCs (0.0026%) (Lalvani *et al.*, 1997), it is known to produce erratic results especially when whole cells are used as stimulators (Pass *et al.*, 1998). This has negative implications for selecting an assay where accurate sequential monitoring of EBV-specific immunity is crucial because treatment will be guided by changes in EBV immunity.

Intracellular cytokine staining (ICS) is another technique that can be used to quantify functional EBV-specific T cells (Kuzushima *et al.*, 1999; Lucas *et al.*, 2004). T cells are cultured briefly *in vitro* with EBV antigen in the presence of Brefeldin A that disables the release of newly synthesized cytokines from the endoplasmic reticulum.

Following cell permeabilization, the accumulation of intracellular cytokines can be labelled by fluorochrome-labelled antibodies and detected by flow cytometry.

ICS has several advantages over ELISpot for detection of EBV-specific T cells. The reliability of ICS over ELISpot has been proven in several studies. Kuzushima *et al* showed that on average EBV-specific T cell responses stimulated by autologous LCLs and detected by ICS were four times higher than those seen with ELISpot (Kuzushima *et al.*, 1999). This apparent increase in sensitivity with ICS over ELISpot was also seen in data determining the frequency of CMV-specific CD4⁺ T cells in healthy individuals and HIV patients (Waldrop *et al.*, 1997) and in determining the CD8⁺ T cell response to restimulation with tetanus toxoid (Tassinon *et al.*, 2005). Another advantage of ICS is that it uses flow cytometry as a platform for analysis. This means it is highly amenable to standardization (Landay, 2004) and can be readily combined with cell surface activation antigen marker phenotyping to enable multi-parameter evaluation of EBV-specific T cells. In addition, flow cytometry is an established technique in many specialist clinical immunology departments and therefore very cost effective in terms of reagents, specialist equipment and staff training. ELISpot, however, cannot be combined with other techniques, requires dedicated instrumentation for enumerating spot-forming cells and is not used routinely in clinical laboratories.

Due to these advantages, ICS was selected as the method of choice for the development of a rapid and functional EBV-specific immuno-assay.

The aims of the work described in this chapter were to develop a rapid and reliable assay that could be used in the clinical setting to enumerate EBV-specific T cell responses in patients with EBV-driven malignancies. The assay was optimized using

EBV sero-positive healthy individuals and investigated several different forms of EBV antigen as stimulators of an EBV-specific T cell response. Autologous EBV-transformed LCLs were initially used as they are easily generated in healthy individuals and express the full complement of EBV latent antigens. However, in patients with EBV-driven malignancies, autologous LCLs take longer to generate *in vitro* and are not always successful. Such a delay is impractical in patients with PTLN, and therefore partially HLA-matched LCLs were investigated as alternatives to autologous LCLs. HLA-restricted EBV peptides were also investigated as stimulators of an EBV-specific T cell response. Peptides can be stored at -80°C and made available for immediate use as required. LCLs, however, require at least 24 hours of *in vitro* culture prior to use as a T cell stimulator.

Each of these sources of EBV antigen was compared to ascertain which was the most effective and reliable at stimulating an EBV-specific T cell response.

Table 3.1: Available assays for enumerating EBV-specific T cells (Adapted from (Kern *et al.*, 2005))

3.2 RESULTS

3.2.1 Quantification of EBV-specific T cell responses using EBV-transformed LCLs as stimulators presenting EBV antigens

EBV-transformed LCLs represent ideal stimulators of an EBV-specific T cell response as they express all of the EBV latent antigens as well as high surface levels of MHC Class I and Class II, making them potent EBV APCs. It was therefore appropriate initially to optimize an EBV-specific assay in healthy sero-positive individuals using autologous LCLs and then investigate the use of HLA-matched LCLs.

3.2.1.1 *Autologous LCLs as stimulators of an EBV-specific T cell response*

IFN- γ production by both CD8⁺ T cells (Callan *et al.*, 2000; Hislop *et al.*, 2001; Hoshino *et al.*, 1999) and CD4⁺ T cells (Amyes *et al.*, 2003) has been used to successfully monitor EBV-specific T cell effector function. However, other cytokines can also contribute to antigen specific T cell-mediated immune responses, including production of TNF- α by CD8⁺ T cells and IL-2 or TNF- α by T_H1 CD4⁺ T cells.

In order to investigate which was the most effective cytokine to monitor for detection of a functional EBV-specific T cell response, PBMCs from sero-positive healthy individuals were stimulated with autologous LCLs, and intracellular staining was performed using a spectrum of anti-cytokine m-Abs. Compilation of the results for six EBV sero-positive healthy individuals (**Figure 3.1**) reveals that IFN- γ production by CD8⁺ T cells was significantly the most reliable indicator of an EBV-specific immune response compared to the background response ($p=0.0022$) with a positive

response detected in all individuals. IFN- γ production from CD4⁺ T cells was also reasonably reliable ($p=0.04$) with a positive response detected in five of the six individuals. Production of all other cytokines by CD8⁺ and CD4⁺ T cells was not consistently positive. Therefore, IFN- γ production from CD8⁺ and CD4⁺ T cells was chosen to demonstrate an EBV-specific T cell response in all subsequent experiments.

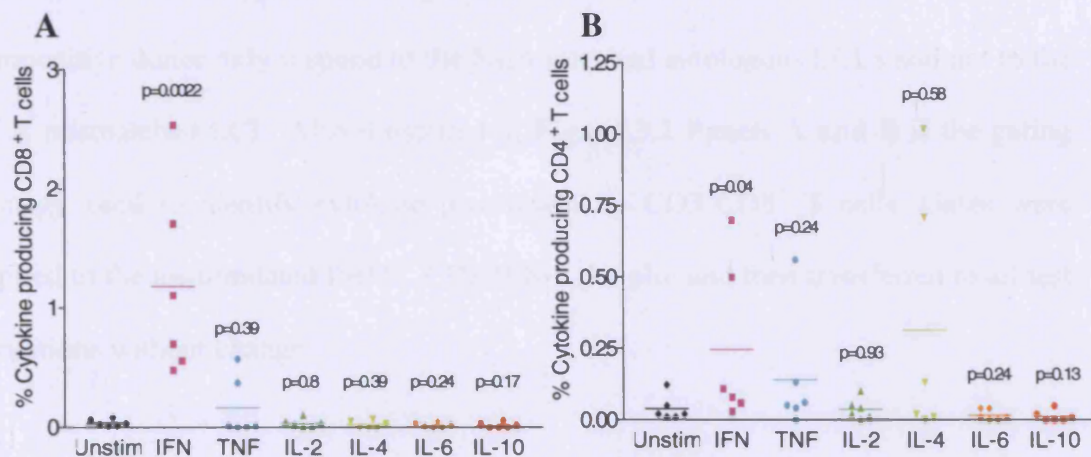


Figure 3.1: Comparison of cytokine production by EBV-specific T cells

PBMCs from 6 different healthy individuals were stimulated by autologous LCLs for 16 hours in the presence of Brefeldin A. EBV-specific T cells were enumerated by measuring cytokine production by CD8⁺ and CD4⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells (**Panel A**) or CD3⁺CD4⁺ (**Panel B**). Horizontal lines represent the mean values. Statistical analysis to compare the cytokine production from CD8⁺ and CD4⁺ T cells was performed using a Mann-Whitney U test. NB: Different scales are used for each graph.

In order to demonstrate the specificity of the assay, PBMCs from an EBV sero-positive and sero-negative healthy individual were stimulated with autologous LCLs. **Figure 3.2** shows that a positive response was elicited from the sero-positive individual (**Panel C**) and no response was seen with the sero-negative individual (**Panel D**), despite a large response on stimulation with PHA. This data confirms the EBV specificity of the T cell response. The results also demonstrate that EBV specific T cell recognition is self HLA restricted because PBMCs from the seropositive donor only respond to the HLA matched autologous LCLs and not to the HLA mismatched LCL. Also illustrated in **Figure 3.2 Panels A and B** is the gating strategy used to identify cytokine production by $CD3^+CD8^+$ T cells. Gates were applied to the unstimulated PBMC CD8/IFN- γ dot plot and then transferred to all test conditions without change.

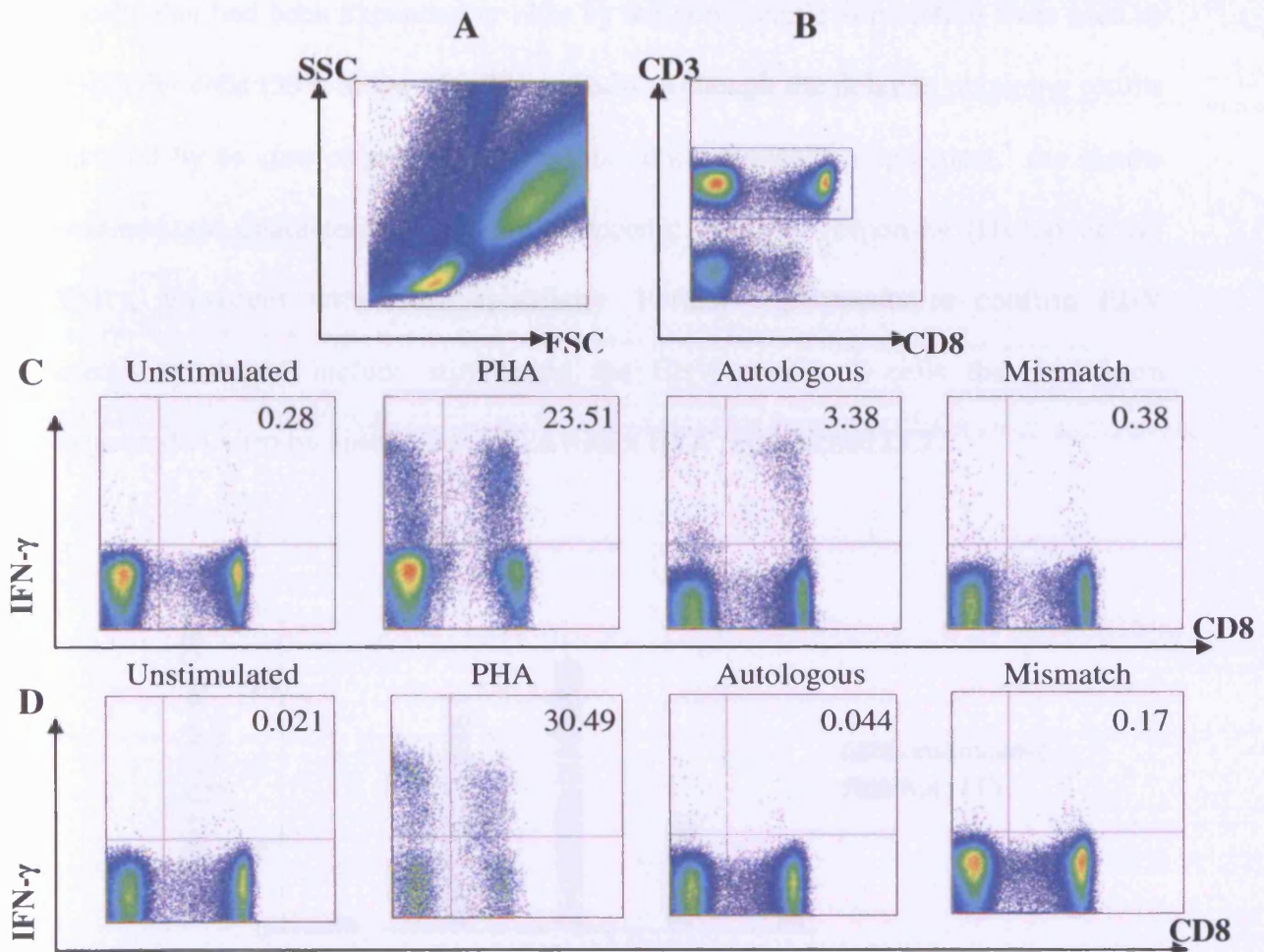


Figure 3.2: A representative example of EBV-specific T cell responses after stimulation with autologous LCLs

PBMCs from an EBV sero-positive healthy individual (**Panels A-C**) and an EBV sero-negative healthy individual (**Panel D**) were stimulated with autologous LCLs and HLA-mismatched LCLs, and compared to unstimulated PBMCs (negative control) and PBMCs stimulated with PHA (positive control).

Lymphocytes were gated from the cell debris on the forward scatter/side scatter plot (**Panel A**). CD3⁺CD8⁺ T cells (**Panel B**) within the lymphocyte population were gated on a second dot plot using the CD3/CD8 plot. IFN- γ production by CD8⁺ T cells was evaluated in a third dot plot (**Panel C/D**). The numbers in each panel represent the percentage of CD3⁺CD8⁺ T cells producing IFN- γ .

The EBV-specific immune response was substantially amplified when EBV-specific T cells that had been expanded *in vitro* by antigen specific stimulation were used as responder cells (33% at Day 16, **Figure 3.3**). Although the delay in obtaining results incurred by *in vitro* expansion makes this option clinically impractical, the results obtained are characteristic of antigen-specific memory responses (Hislop *et al.*, 2001), consistent with EBV specificity. Further experiments to confirm EBV specificity would include stimulating the EBV-specific T cells that had been expanded *in vitro* by autologous LCLs with a HLA mismatched LCL.

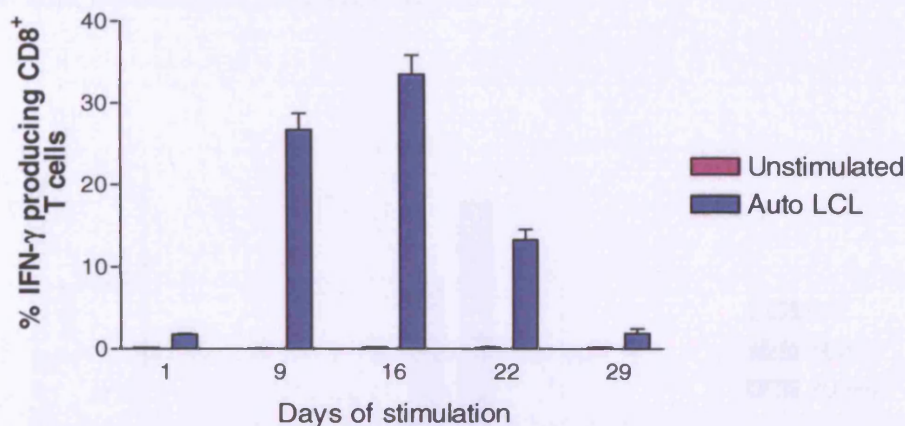


Figure 3.3: Amplification of the EBV-specific T cell response using previously *in vitro* expanded antigen-specific CD8⁺ T cells

EBV-specific T cells were expanded *in vitro* from EBV sero-positive PBMCs by stimulation with irradiated autologous LCLs. PBMCs were initially stimulated on Day 0 with freshly irradiated LCLs as described in **Section 2.2.12**. Every 7 days thereafter cells were restimulated at a ratio of 4 responder cells:1 LCL. IL-2 was added to the culture (20iU/mL final concentration) from Day 14 every 2-3 days. After each restimulation, an aliquot of cells were removed from culture, incubated for 16 hours in the presence of Brefeldin A and analysed using intracellular cytokine staining for IFN- γ producing CD8⁺ T cells. The bars and error bars represent the mean and the sd respectively.

One of the requirements of an assay to enumerate EBV-specific T cell responses in patients with PTLTD is that results are available rapidly so that clinicians can tailor reductions in immunosuppression accordingly. In addition, the total duration of the assay should ideally fit into the normal working hours of a clinical laboratory. **Figure 3.4** compares EBV-specific T cell responses detected in PBMCs from a representative healthy individual after different periods of antigen stimulation. When PBMCs were stimulated with autologous LCLs, 40 hours incubation produced the maximal EBV-specific T cell response (6.3%), however, 16 hours (overnight) incubation also gave a clear positive result (4.8%) above the backgrounds of 0.1% with unstimulated cells and 0.2% with mismatched negative controls.

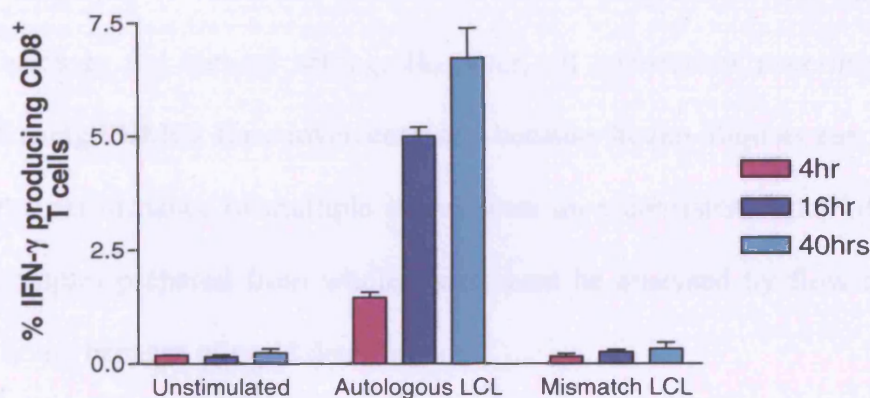


Figure 3.4: Experiments to determine the optimal assay duration

PBMCs from a healthy individual were stimulated for 4, 16 and 40 hours respectively with autologous LCLs or an HLA-mismatched LCL, in the presence of Brefeldin A. After the incubation periods, PBMCs were removed and analysed using intracellular cytokine staining for IFN- γ producing CD8⁺ T cells. The bars and error bars represent the mean and the sd respectively.

Experiments were performed to determine the optimal ratio of responder cells and stimulator LCLs to use, and to evaluate feasibility of using whole blood as responder cells. PBMCs are traditionally used as responders in the enumeration of EBV-specific T cell responses. However, using whole blood would significantly reduce setting up time for the assay by circumventing the need to isolate PBMCs.

Serum-free whole blood was prepared and red cells were lysed according to the method described in chapter 2, adjusted to 1×10^6 cells/ml and stimulated with different ratios of autologous LCLs for 16 hours. EBV-specific T cell responses were compared to unstimulated controls. **Figure 3.5** shows that equivalent EBV-specific T cell responses were detected using PBMCs or red cell lysed serum-free whole blood. In addition, the responses were comparable for both PBMCs and whole blood from a 2:1 to 1:2 responder to stimulator ratio. These results demonstrate that whole blood could be used in the clinical setting. However, all subsequent experiments were performed using PBMCs for convenience and because frozen aliquots can be stored enabling the performance of multiple experiments on a consistent batch of cells. In addition, samples prepared from whole blood must be analysed by flow cytometry within 48 hours because of rapid deterioration.

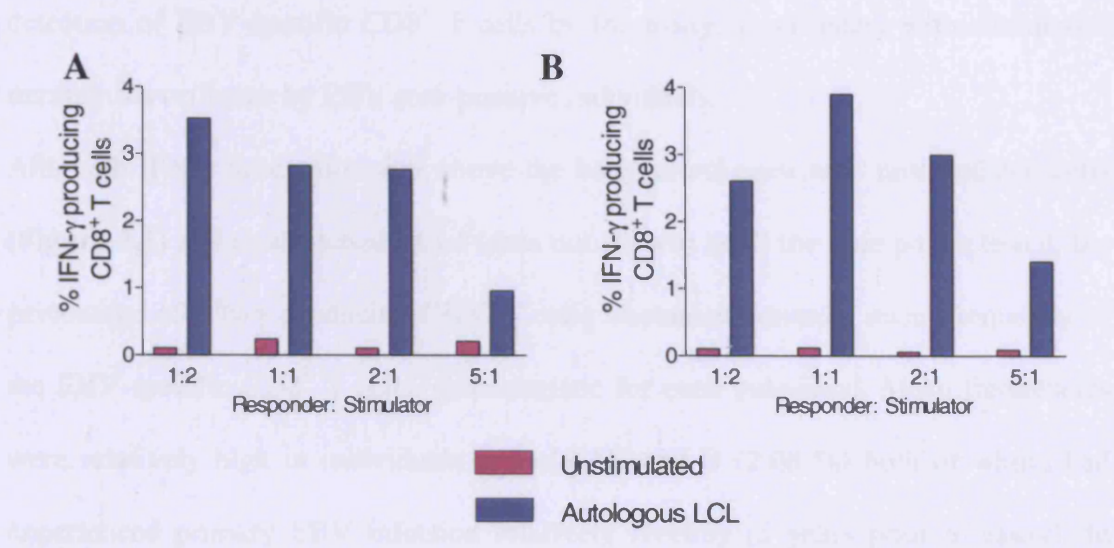


Figure 3.5: Comparison of EBV-specific T cell responses detected using PBMCs (A) and red cell lysed whole blood (B) and different ratios of LCLs as stimulators

PBMCs (A) or serum-free whole blood (B) from a healthy individual, were adjusted to $1 \times 10^6/\text{mL}$ and stimulated with different ratios of autologous LCLs for 16 hours in the presence of Brefeldin A. Unstimulated serum-free whole blood or PBMCs were used as a negative control. EBV-specific T cells were enumerated by measuring the IFN- γ production from CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells.

A crucial consideration in evaluating and optimising the assay was determining its consistency over time. EBV sero-positive healthy individuals are able to control EBV driven proliferation due to continuous presence of effective EBV specific immunity. To evaluate whether IFN- γ production after stimulation with autologous LCLs consistently detected EBV immunity, EBV-specific T cell responses from four EBV sero-positive healthy individuals were serially monitored over a 1.5 to 2 year period. **Figure 3.6 Panel A** demonstrates that EBV-specific responses were detected at all the time points tested in each of the four individuals. This data therefore confirms that

detection of EBV-specific CD8⁺ T cells by the assay is consistent with continuous immune surveillance by EBV sero-positive individuals.

Although IFN- γ production was above the background seen with unstimulated cells (**Figure 3.6**) and mismatched LCLs (data not shown) at all the time points tested, the percentage of IFN- γ producing CD8⁺ T cells fluctuated around a mean frequency of the EBV-specific CD8⁺ T cells, characteristic for each individual. Mean frequencies were relatively high in individuals A (3.19 %) and B (2.08 %) both of whom had experienced primary EBV infection relatively recently (5 years prior to assay). In contrast individuals C and D had a relatively low means (1.06 % and 1.20 %, respectively) and had experienced primary EBV infection 30-40 years prior to the assay.

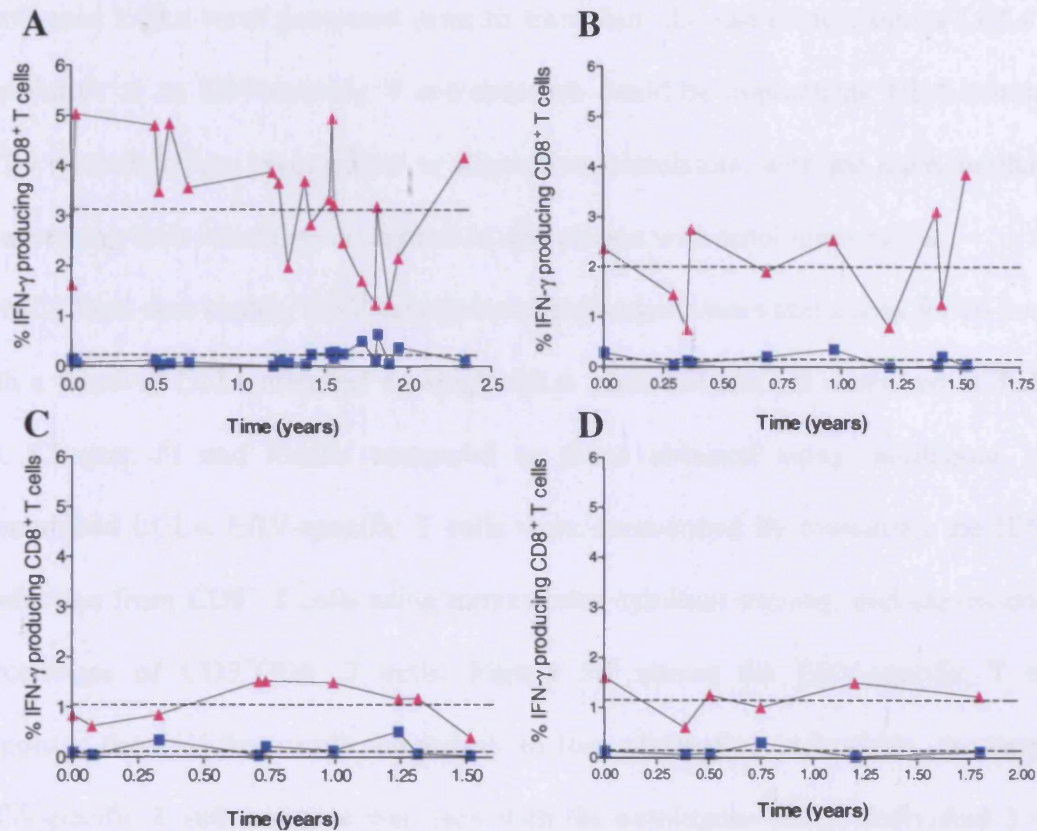


Figure 3.6: Serial monitoring of EBV sero-positive healthy individuals to determining the consistency with which the assay detects EBV-specific T cell responses

PBMCs from four healthy EBV sero-positive individuals (**Panels A-D**) were stimulated for 16 hours with autologous LCLs (\blacktriangle) and compared to an unstimulated control (\blacksquare) and a mismatched LCL control (data not shown). The frequency of EBV-specific T cells was analysed by intracellular cytokine staining and flow cytometry after stimulation. EBV-specific T cells were expressed as a percentage of CD3⁺CD8⁺ T cells. The dotted lines show the mean EBV-specific T cell responses from stimulation with autologous LCLs and the unstimulated control.

3.2.1.2 HLA-matched LCLs as stimulators of an EBV-specific T cell response

Generating EBV-transformed LCLs from PBMCs takes approximately 3-4 weeks of *in vitro* culture, and is usually successful in 100% of healthy individuals. However, in patients with PTLD who are often severely immunosuppressed, generating autologous LCLs takes longer and has a lower success rate. Therefore, unless

autologous LCLs were generated prior to transplant the use of autologous LCLs as stimulators of an EBV-specific T cell response could be impractical. HLA-matched LCLs were therefore investigated as alternative stimulators, with the main emphasis on assessing their reliability compared to stimulation with autologous LCLs.

PBMCs from five healthy EBV sero-positive individuals were stimulated for 16 hours with a panel of LCLs matched on single HLA class I alleles (as described in **Table 2.3**, Chapter 2) and results compared to those obtained using autologous and mismatched LCLs. EBV-specific T cells were enumerated by measuring the IFN- γ production from CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells. **Figure 3.7** shows the EBV-specific T cell responses detected from each individual. In four of the five individuals, the largest EBV-specific T cell response was seen with the autologous LCLs. Individual 3 did not respond to stimulation with the autologous LCL in this experiment, despite having a positive response to the same LCL on several other occasions. A possible explanation for this negative result is *Mycoplasma* contamination of the LCL. In individual 1, large EBV-specific T cell responses were seen on stimulation with LCLs matched on HLA-A*0201 (**Panel 1a**) and HLA-B*1801 (**Panel 1b**) alleles alone compared to weaker responses seen on stimulation with HLA-A*0101 (**Panel 1a**) and B*4402 (**Panel 1b**) matched LCLs. Similar results were also observed in individual 5, where the largest CD8⁺ T cell responses were restricted through HLA-A*0201 (**Panel 5a**) and HLA-B*0702 alleles (**Panel 5b**).

These results suggest HLA-restricted immunodominance, a phenomenon which has been described in the context of cytomegalovirus (CMV) (Lacey *et al.*, 2003), EBV

(Hollsberg, 2002) and HIV-1 (Kiepiela *et al.*, 2004). The patterns of immunodominance were not consistent amongst the individuals tested. Individuals 1 and 5 showed immunodominant responses restricted through HLA-A*0201, but individuals 2, 3 and 4 showed no patterns of HLA-restricted immunodominance, despite all possessing the HLA-A*0201 allele. All the responses in individuals 2 and 4 were weak and individual 3 had responses of similar strength restricted through both HLA-A and HLA-B alleles.

Therefore, in order for a panel of LCLs matched on single HLA alleles to enumerate an EBV-specific T cell response successfully, it is essential that all single HLA-A and HLA-B allele are screened in each individual to avoid missing a positive response (e.g. selecting LCLs matched on HLA-A*0101 alone in individual 1 would have failed to detect immunity to EBV).

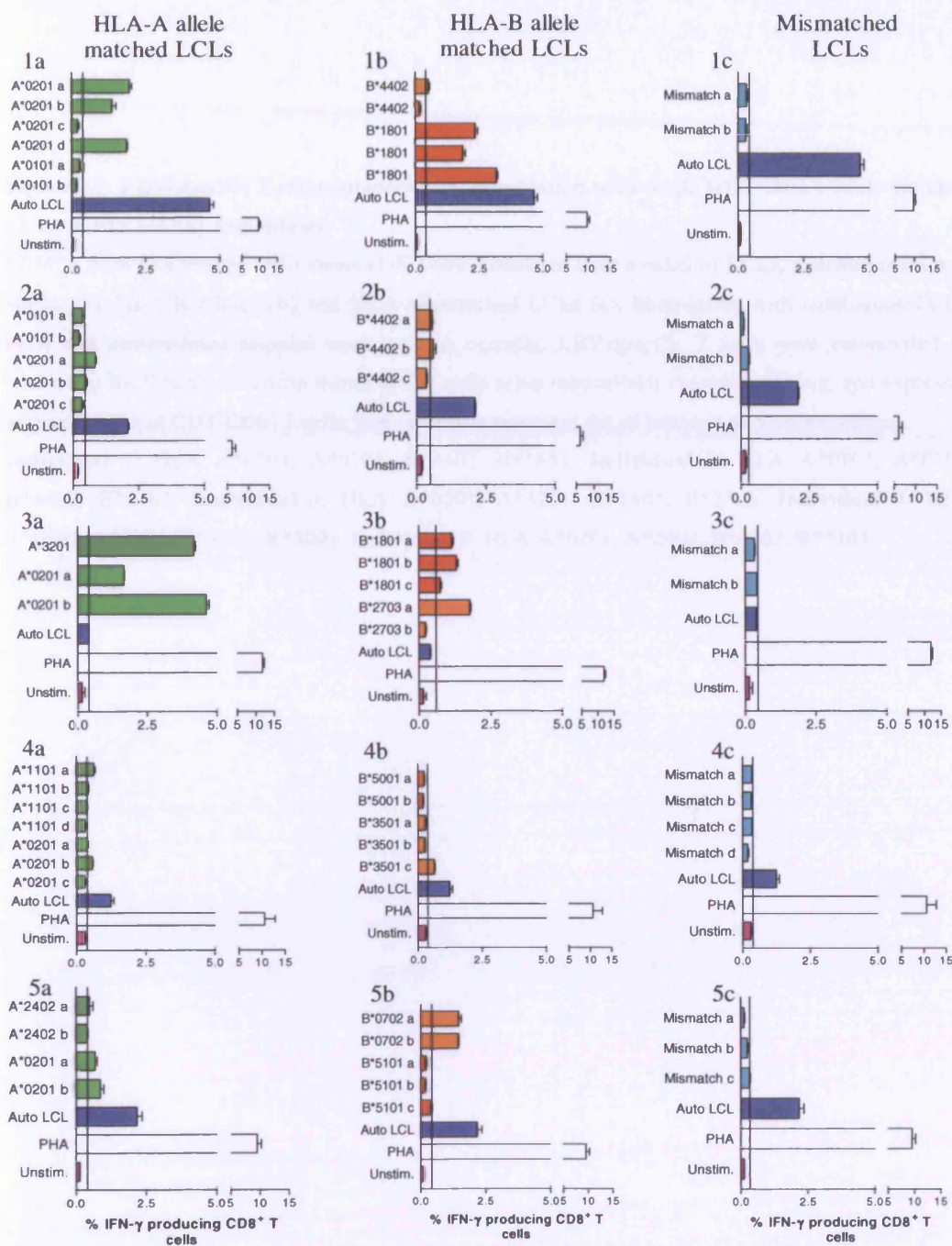


Figure 3.7: EBV-specific T cell responses after stimulation with single HLA class I allele matched LCLs in five healthy individuals

PBMCs from five healthy individuals (1-5) were stimulated with a panel of LCLs, matched at HLA-A alleles (a), HLA-B alleles (b) and HLA-mismatched LCLs (c). Stimulation with autologous LCLs, PHA and unstimulated samples were used as controls. EBV-specific T cells were enumerated by measuring the IFN- γ production from CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells. The error bars represent the sd between duplicate samples.

Individual 1: HLA A*0201, A*0101, B*4402, B*1801. **Individual 2:** HLA A*0201, A*0101, B*4402, B*5701. **Individual 3:** HLA A*0201, A*3201, B*1801, B*2703. **Individual 4:** HLA A*0201, A*1101, B*3501, B*5001. **Individual 5:** HLA A*0201, A*2402, B*0702, B*5101.

Figure 3.7 reveals variations in the magnitude of EBV-specific T cell responses detected from different LCLs expressing the same single shared HLA class I allele. This is illustrated by individual 1 who had a positive response to stimulation with only three out of four different LCLs matched on HLA-A*0201, despite demonstrating an immunodominant EBV-specific response restricted through HLA-A*0201. These false negative results were particularly frequent in individuals where EBV-specific T cell responses were weak and no patterns of immunodominance were seen. For example individual 4 had a positive response to 2 out of 3 different LCLs matched on HLA-A*0201 and HLA-A*1101 and only 1 out of 3 different LCLs matched on HLA-B*3501. A summary of the total number of positive responses of each HLA allele for each individual is shown in **Table 3.1**. Possible explanations for this variation are loss of HLA molecule expression and therefore EBV epitope presentation on the surface of some LCLs, an observation which had been seen in Burkitt lymphoma cell lines (Torsteinsdottir *et al.*, 1988; Masucci *et al.*, 1987), or that some LCLs have insufficient surface concentrations of HLA:peptide complexes necessary to trigger T cell activation (Hill *et al.*, 1995).

	<i>Individual 1</i>	<i>Individual 2</i>	<i>Individual 3</i>	<i>Individual 4</i>	<i>Individual 5</i>
Autologous LCL	Y	Y	N	Y	Y
A*0201 LCL a	Y	Y	Y	Y	Y
A*0201 LCL b	Y	Y	Y	N	Y
A*0201 LCL c	N	N	nd	Y	nd
A*0201 LCL d	Y	nd	nd	nd	nd
A*0101 LCL a	N	N			
A*0101 LCL b	N	N			
A*3201 LCL			Y		
A*2401 LCL a					Y
A*2401 LCL b					Y
A*1101 LCL a				Y	
A*1101 LCL b				Y	
A*1101 LCL c				N	
A*1101 LCL d				N	
B*4402 LCL a	Y	Y			
B*4402 LCL b	N	Y			
B*4402 LCL c	nd	N			
B*1801 LCL a	Y		Y		
B*1801 LCL b	Y		Y		
B*1801 LCL c	Y		Y		
B*2702 LCL a			Y		
B*2702 LCL b			N		
B*3501 LCL a				Y	
B*3501 LCL b				N	
B*3501 LCL c				Y	
B*5001 LCL a				N	
B*5001 LCL b				N	
B*5101 LCL a					N
B*5101 LCL b					N
B*5101 LCL c					N
B*0702 LCL a					Y
B*0702 LCL b					Y
Total number of positive responses	7/11	4/8	7/8	6/12	6/9

Table 3.2: Summary of results obtained for five healthy EBV sero-positive individuals using single HLA class I allele matched LCLs as stimulators

The mean EBV-specific T cell responses after stimulation with a panel of HLA-matched LCLs were compared to mismatched LCLs, in 5 healthy individuals. A positive result was identified when the mean value exceeded the highest mean value obtained from at least 2 different mismatched LCLs.

Y = positive result. N= negative result. nd = not done.

Individual 1: HLA A*0201, A*0101, B*4402, B*1801. **Individual 2:** HLA A*0201, A*0101, B*4402, B*5701. **Individual 3:** HLA A*0201, A*3201, B*1801, B*2703. **Individual 4:** HLA A*0201, A*1101, B*5001, B*3501. **Individual 5:** HLA A*0201, A*2402, B*5101, B*0702.

The implications for using a panel of single HLA class I allele matched LCLs as stimulators of an EBV-specific T cell response in a clinical assay are twofold. Firstly, several different LCLs sharing the same allele need to be tested to ensure a positive response is detected. Secondly, the LCLs need to represent all the HLA-A and HLA-B alleles expressed by the responder in order to accommodate biases in HLA-restriction due to immunodominance. Many different LCLs are therefore required, necessitating large numbers of PBMCs. This is illustrated by individual 2 in which a positive EBV-specific T cell response was detected from only four of the eight LCLs used in the panel. This panel with the appropriate positive and negative controls required 11×10^6 PBMCs to detect a positive response. This approach would be impractical for patient samples where the PBMC numbers are often limited.

In contrast, autologous LCLs almost always gave much higher responses than the LCLs matched on single HLA class I alleles. In individuals where autologous LCLs fail to detect a positive EBV-specific T cell response (individual 3) or where they are not available, LCLs matched on multiple HLA class I alleles may provide an alternative. This was evaluated with patient PBMC samples and results are presented in Chapter 4.

3.2.1.3 HLA class I restricted EBV peptides as stimulators of an EBV-specific T cell response

Previous studies have identified a large number of EBV peptide epitopes restricted through a wide range of HLA class I alleles (**Tables 2.1 and 2.2**) and a hierarchy of T cell responses to both latent cycle proteins (reviewed by (Rickinson and Moss, 1997)

and lytic cycle proteins (Pudney *et al.*, 2005) has been detected in healthy individuals. Studies to enumerate EBV-specific T cells include detection of IFN- γ release from epitope-specific CD8⁺ T cells following peptide stimulation (Lalvani *et al.*, 1997; Tan *et al.*, 1999; Yang *et al.*, 2000a) and use of fluorochrome-labelled HLA class I-peptide tetramers to detect EBV specific T cells in healthy individuals (Altman *et al.*, 1996; Tan *et al.*, 1999) paediatric transplant patients (Falco *et al.*, 2002) and patients with Hodgkin's lymphoma receiving adoptive T cell therapy (Bollard *et al.*, 2004). In all of these studies, only CD8⁺ T cell responses to single immunodominant EBV epitopes restricted through common HLA class I alleles have been examined. To be of utility for routine clinical assays it would be necessary to use pools of EBV peptide epitopes to enumerate EBV-specific T cell responses restricted by a wide spectrum of HLA class I alleles.

Initial experiments to optimize peptide stimulation conditions were performed using HLA-A*02 EBV sero-positive individuals as a large number of HLA-A*02 restricted peptide epitopes have been characterised. 10 μ g/mL final concentration of each EBV peptide within the HLA-A*02 restricted peptide pools stimulated detectable EBV-specific T cell responses (**Figure 3.8, Panel A**). Higher final concentrations were not tested as the DMSO concentration in each peptide pool would exceed the threshold known to cause cell death (1% final concentration). Synthetic peptides purified to 80% or 95% were tested to assess whether costs could be reduced by using preparations of lower purity. Although peptides of 80% purity were able to induce responses, peptides of 95% purity produced higher responses (**Figure 3.8, Panel B**). Optimal assay duration was shown to be 16 hours (**Figure 3.8, Panel C**) and therefore

consistent with the data using autologous LCLs (**Figure 3.4**). These set of conditions were subsequently used for all further experiments.

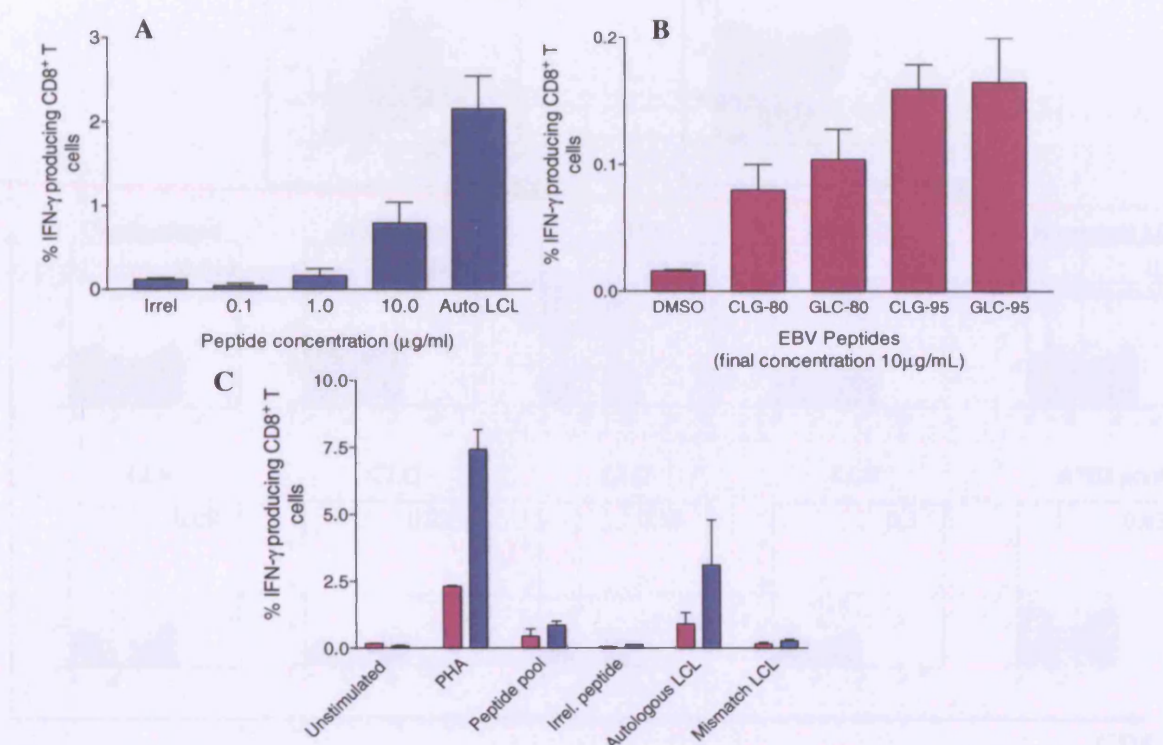


Figure 3.8: Optimisation of the ICS assay to enumerate EBV-specific T cell responses in PBMCs stimulated with synthetic peptides

Panel A: PBMCs were stimulated with peptide pools containing 15 EBV HLA-A*02 restricted peptides each at 0.1, 1 and 10 μg/l final concentration, or an autologous LCL for 16 hours. PBMCs stimulated with a combination of an EBV peptide restricted by an irrelevant HLA class I allele (final concentration 10 μg/l) and DMSO were used as a negative control. **Panel B:** PBMCs were stimulated with 80% (CLG-80) and 95% (CLG-95) pure CLGGLLTMV, a latent epitope restricted through HLA-A*02, and 80% (GLC-80) and 95% (GLC-95) pure GLCTLVAML, a lytic epitope restricted through HLA-A*02, for 16 hours. **Panel C:** PBMCs were stimulated with HLA-A*02 restricted EBV-peptide pools (15 peptides all at 10 μg/ml final concentration) for 4 (■) and 16 (■) hours respectively. EBV-peptide pools restricted through irrelevant HLA alleles (Irrelevant peptide) and mismatched LCLs were used as negative controls. EBV-specific T cells were enumerated by measuring the IFN-γ production from CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells. The bars and error bars represent the mean and the sd respectively, from at least 3 healthy HLA-A*02 EBV sero-positive individuals.

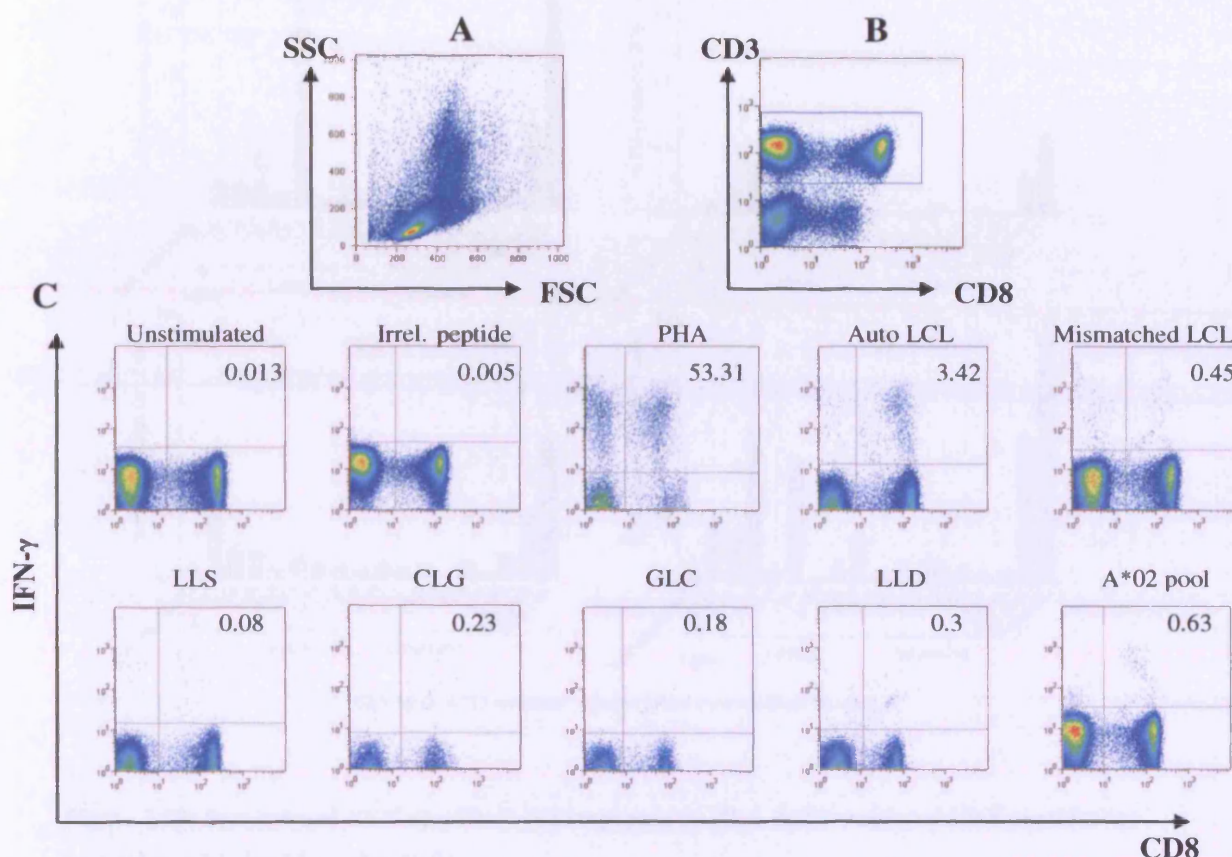
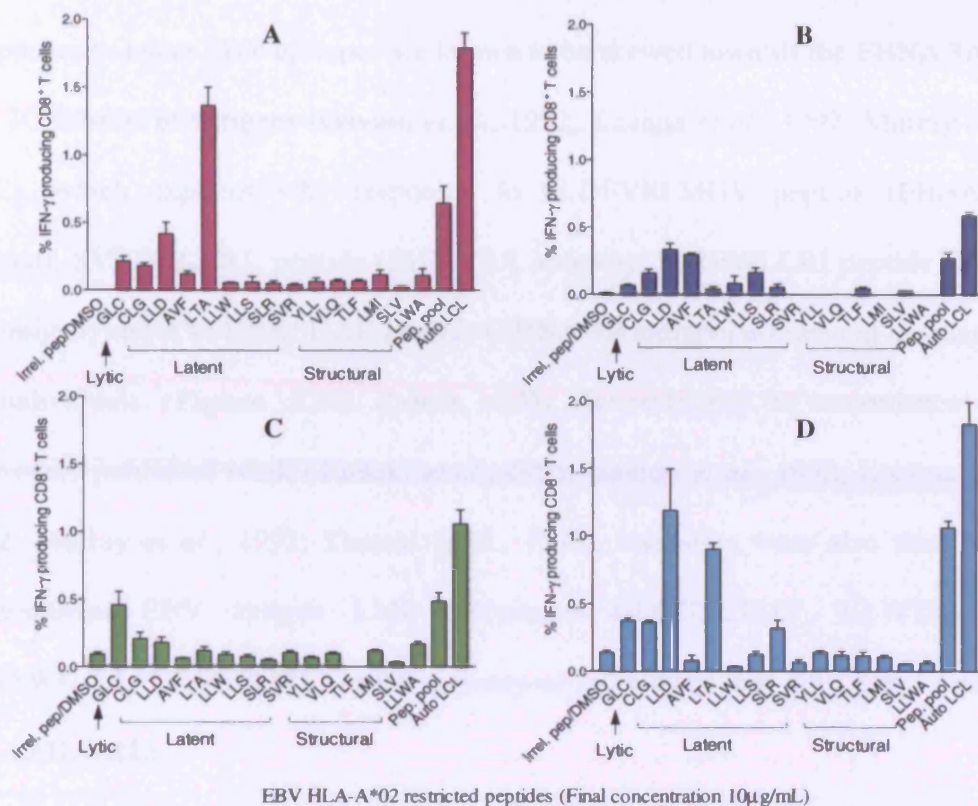


Figure 3.9: Example of EBV-specific T cell responses seen on stimulation with individual and pools of HLA-A*02 restricted EBV peptides

PBMCs from a healthy individual were stimulated with 4 individual HLA-A*02 EBV peptides and a pool of 15 HLA-A*02 restricted EBV peptides at 10 μ g/ml final concentration. An autologous LCL and PBMCs stimulated with PHA were used as positive controls. A mismatched LCL, unstimulated PBMCs and PBMCs stimulated with an EBV-peptide restricted through an irrelevant HLA allele (Irrel. Peptide) were used as negative controls. The names of the EBV peptides refer to the first three letters of the epitope. Lymphocytes were gated from the cell debris on the forward scatter/side scatter plot (panel A). CD3⁺CD8⁺ T cells (panel B) were gated on a second dot plot using the CD3/CD8 plot. The plots were then gated for IFN- γ in a third dot plot (panel C). The numbers in each panel represent the percentage of CD3⁺CD8⁺ T cells producing IFN- γ .



EBV HLA-A*02 restricted peptides (Final concentration 10 μ g/mL)

Figure 3.10: Spectrum of EBV-specific T cell responses to HLA-A*02 restricted EBV peptides in four HLA-A*02 healthy individuals

PBMCs from four HLA-A*02 healthy individuals (A–D) were stimulated with 15 individual A*02 EBV peptides (final concentration, 10 μ g/mL) and a pool of 15 HLA-A*02 restricted peptides (Pep. Pool). EBV-peptides restricted through irrelevant HLA alleles (Irrel. Peptide/DMSO) and autologous LCLs were used as negative and positive controls respectively. EBV-specific T cells were enumerated by measuring the IFN- γ production by CD8 $^{+}$ T cells using intracellular cytokine staining, and expressed as percentages of CD3 $^{+}$ CD8 $^{+}$ T cells. The names of the EBV peptides refer to the first three letters of the epitope. The bars and error bars represent the mean and the sd respectively.

Individual HLA-A*02-restricted EBV peptides were then used to stimulate EBV-specific T cell responses with PBMCs from four healthy HLA-A*02 individuals (Figures 3.9 and 3.10).

The results show no consistent patterns in responses from the four individuals. Responses to latent EBV epitopes are known to be skewed towards the EBNA 3A, 3B and 3C subsets of antigens (Gavioli *et al.*, 1992; Khanna *et al.*, 1992; Murray *et al.*, 1992), which explains why responses to LLDFVRFMGV peptide (EBNA 3C antigen), SVRDLARL peptide (EBNA 3A antigen), SLREWLLRI peptide (EBNA 3C antigen) and AVFDRKSDAK peptide (EBNA 3B antigen) are seen in the majority of individuals (**Figure 3.10, Panels A-D**). Interestingly, in accordance with previously published work (Tamaki *et al.*, 1995; Gavioli *et al.*, 1992; Khanna *et al.*, 1992; Murray *et al.*, 1992; Tamaki *et al.*, 1995), responses were also seen to the subdominant EBV antigen LMP-2 (epitopes CLGGLLTMV, LLWTLVVLL, LLSAWILTA, LTAGFLIFL) in the majority of individuals, but not LMP-1 (epitope YLLEMLWRL).

Previous work has shown that in healthy individuals, most EBV-specific CD8⁺ T cell responses are directed against intermediate or early lytic proteins: BZLF1, BRLF1 and BMLF1 (Khanna *et al.*, 1992; Callan *et al.*, 1998b; Landais *et al.*, 2005; Murray *et al.*, 1992; Saulquin *et al.*, 2000; Tamaki *et al.*, 1995). Therefore, although only one lytic epitope, GLCTLVAML (BMLF-1 antigen), was available for testing, surprisingly small responses were seen and only in two individuals. Weak responses to structural proteins gp85 (epitopes: LMIIPLINV, TLFIGSHVV and SLVIVTTFV) and gp350 (epitope VLQWASLAV) were also seen in three of individuals (Khanna *et al.*, 1999).

This data demonstrates the limitations of using single EBV-peptides to enumerate EBV-specific T cells in a clinical assay. Firstly, responses to many of the known immunodominant peptides could not be detected and secondly, no consistent patterns

of response were seen for the same HLA-A*02 epitopes amongst the four HLA-A*02 individuals tested. A possible explanation for these results is that the individuals with lower responses to immunodominant EBV peptides (**Figure 3.10, Panels B and C**) both acquired primary EBV 30-40 years prior to the assay. Precursor frequencies of EBV-specific T cells in these individuals would be expected to be low and therefore may be below the level required for detection by ICS. In order to determine a positive EBV-specific T cell response, all peptides for each HLA class I allele would need to be tested for each person. Testing each peptide individually requires 1×10^6 PBMCs per peptide and thus enumerating an EBV-specific T cell response from individuals with common HLA alleles with this approach could require up to 25×10^6 PBMCs. This is clearly impractical in a clinical setting and therefore the use of pools of peptides to stimulate PBMCs was investigated. A cocktail of the 15 HLA-A*02 binding epitopes stimulated a detectable T cell response in all four healthy HLA-A*02 individuals tested (**Figure 3.10**). Interestingly, the responses to HLA-A*02 peptide pools did not equate to the sum of the responses to individual peptides in any of the individuals - this may be due to competition between peptides for binding to HLA-A*02. Peptides with higher affinity are likely to competitively inhibit binding of peptides with lower affinity thus diminishing the magnitude of the T cell response stimulated by the weaker HLA-A*02 binding peptides.

It was hypothesised that a combination of peptide pools tailored to an individual's HLA type might generate EBV-specific responses comparable to those achieved with an autologous LCL. In each peptide pool, DMSO toxicity was avoided by ensuring that the final DMSO concentration did not exceed 1% of the culture media. **Figure 3.11** shows EBV-specific T cell responses produced by stimulation with peptide pools

and LCLs in a representative healthy individual, and details the gating strategy used for the flow cytometry analysis. The negative control is PBMCs stimulation with an irrelevant peptide/DMSO (**Panel D**). The HLA-A*02 peptide pool produced the largest response (**Panel G**), while weaker responses were produced by the HLA-B*4402 (**Panel H**) and HLA-B*1801 pools (**Panel I**). The EBV-specific response was further increased by combining the three pools (**Panel J**, 0.85 %) but did not equate to the sum of the three individual pools restricted through single HLA alleles ($0.65 \% + 0.3 \% + 0.39 \% = 1.34 \%$). The EBV-specific T cell response from a combination of peptide pools restricted to 3 HLA class I alleles was significantly less (0.85 %) than the response with the autologous LCL (5.0 %).

Six healthy EBV sero-positive individuals were screened with tailored peptide pools for each HLA class I allele and a pool containing both HLA-A and HLA-B restricted peptides. Results are summarized in **Figure 3.12**. In 5 out of the 6 individuals tested, use of a cocktail of peptides comprising epitopes restricted by several HLA-A and HLA-B alleles stimulated detectable EBV-specific T cell responses (**Figure 3.12 Panels A, C, D, E, F**). All positive responses were, however, consistently weaker than those observed after stimulation with autologous LCLs. In addition, the response for the combined peptide pools restricted through different HLA alleles consistently gave a lower response than expected from the sum of the single HLA class I allele restricted pools in all individuals tested - this may be due to competition between peptides for binding to HLA class I molecules.

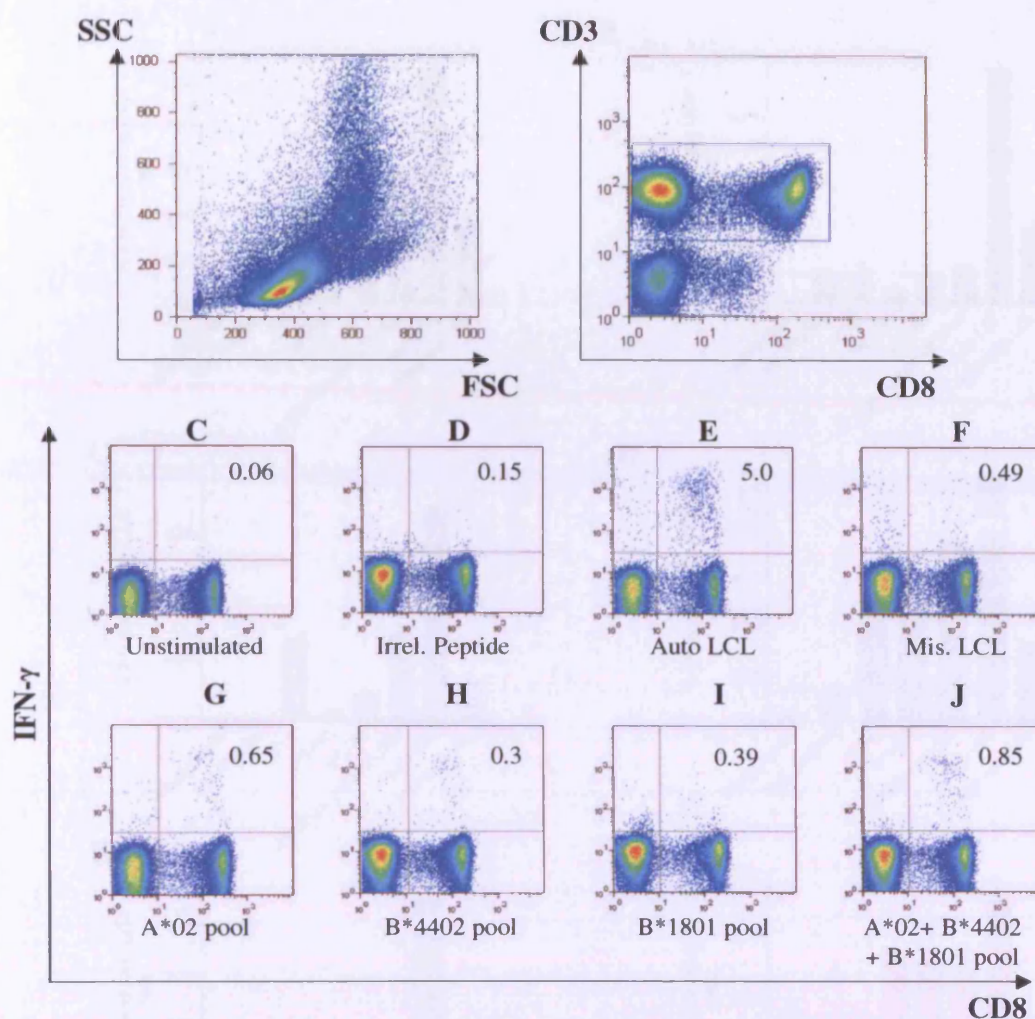


Figure 3.11: Example of EBV-specific T cell responses seen on stimulation with pools EBV peptides

PBMCs from a healthy individual (HLA A*0201, A*0101, B*4402, B*1801) were stimulated with HLA-restricted EBV peptide pools (**panels G-J**). The final concentration of each peptide used was 10 μ g/ml. Responses were compared to an unstimulated sample (**panel C**), PBMCs stimulated with a peptide restricted to an irrelevant HLA allele (Irrel. Peptide) (**panel D**, DMSO concentration was equivalent to the highest concentration in the peptide pools), an autologous LCL (**panel E**) and a mismatched LCL (**panel F**). Lymphocytes were gated from cell debris on the forward scatter/side scatter plot (**panel A**). CD3⁺ve were gated on a second dot plot using the CD3/CD8 plot (**panel B**). IFN- γ ⁺ cells were then gated and plots show results for the total CD3⁺ T cells (**panel C-J**). The numbers in each panel represent the percentage of CD3⁺CD8⁺ T cells producing IFN- γ .

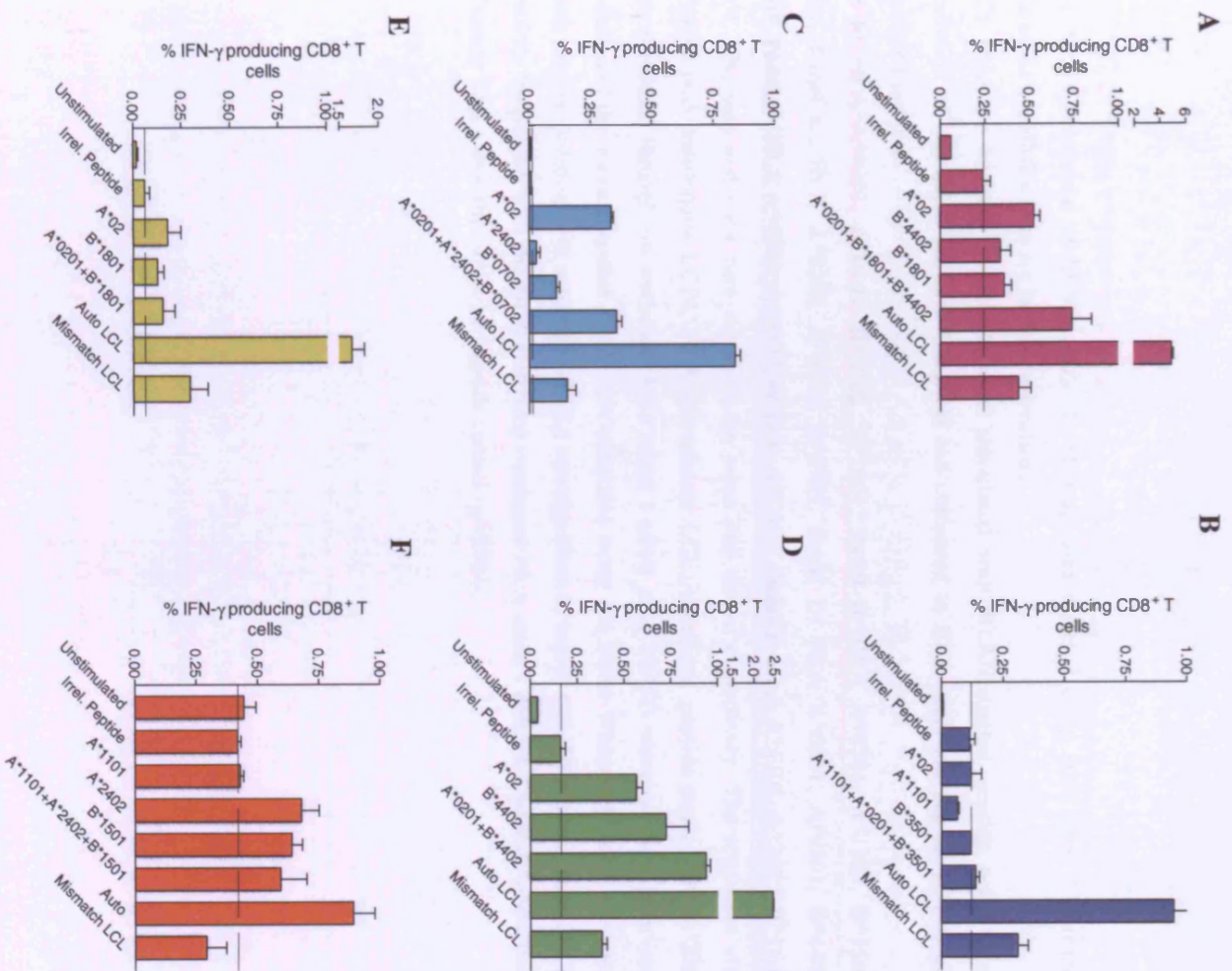


Figure 3.12: Comparison of EBV-specific T cell responses detected using HLA class I restricted peptide pools in PBMCs from six healthy individuals

PBMCs from six healthy individuals were stimulated with HLA-restricted peptide pools (final concentration of individual peptides 10µg/ml) and compared to HLA-irrelevant peptide/DMSO and unstimulated samples.

Panel A: HLA A*0201, A*0101, B*4402, B*1801, **Panel B:** HLA A*0201, A*1101, B*3501, B*5001, **Panel C:** HLA A*0201, A*2402, B*0702, **Panel D:** HLA A*0201, A*0101, B*4402, B*5701, **Panel E:** HLA A*0201, A*3201, B*1801, B*2703, **Panel F:** HLA A*1101, A*2402, B*1501, B*5601. The bars and error bars represent the mean and the sd respectively. The responses after stimulation with autologous LCLs, HLA-mismatched LCL, combined peptide pools and an EBV peptide restricted through an irrelevant HLA class I allele plus DMSO were collated from each individual and the mean response from each compared using the Mann-Whitney U test. The mean response after stimulation with autologous LCLs was significantly higher than HLA-mismatched LCLs ($p=0.0022$). Responses with the HLA-restricted combined HLA class I restricted peptide pools were significantly higher than the irrelevant peptide control ($p=0.026$).

The individual who failed to show a positive response (**Panel B**) in this set of experiments had a positive response to the HLA-A*02 peptide pool in previous experiments (**Figure 3.10, Panel B**), and therefore these results can probably be attributed to an abnormally high background response from the negative control peptide.

A concern with use of peptides to stimulate responses is that the assay relies on peptide presentation by APCs within the population of PBMCs. APC numbers may vary between individuals and there may also be variation in co-stimulatory potential. Waldrop *et al* (Waldrop *et al.*, 1998) originally showed that exogenous costimulation with CD28 mAbs increased the observed frequencies of cytokine-producing CD4⁺ T cells in response to a CMV antigen by 50-100% but had no stimulatory effect alone. More, recently anti-CD28 mAb has been used as an exogenous costimulatory molecule in flow-cytometry based intracellular cytokine assays to quantify HIV-specific CD8⁺ T cell responses to novel HIV (Betts *et al.*, 2001) and CMV (Sylwester *et al.*, 2005) epitopes. Preliminary experiments were therefore performed to investigate the impact of providing additional co-stimulation with anti-CD28 mAb in healthy individuals, but no augmentation of EBV-specific T cell responses was observed (data not shown).

3.3 DISCUSSION

This chapter discusses the optimisation of an assay to enumerate EBV-specific T cell responses using multiparameter flow cytometry. Optimisation was performed using PBMCs from EBV sero-positive healthy individuals and evaluated various forms of EBV antigen as stimulators.

Interferon- γ production from CD8⁺ T cells was shown to be the most consistently positive cytokine at demonstrating a functional EBV-specific T cell response after stimulation with autologous LCLs. This data is consistent with previous work which showed that the large expansion of EBV-specific CD8⁺ T cells seen in patients with infectious mononucleosis has effective cytolytic function (Callan *et al.*, 2000; Steven *et al.*, 1996) and that a subpopulation release IFN- γ in response to EBV antigen (Callan *et al.*, 2000; Hoshino *et al.*, 1999). These functions have also been demonstrated in the memory EBV-specific CD8⁺ T cells from healthy EBV sero-positive individuals (Hislop *et al.*, 2001) suggesting that these cells play a protective role in the immune response to EBV. Two further studies have shown that the level of expansion of activated T cells directly correlates with the severity of clinical symptoms in patients with IM (Silins *et al.*, 2001; Williams *et al.*, 2004) supporting the view that flu-like symptoms of IM, which are indicative of a beneficial immune response, are a direct sequelae of the expansion of activated EBV-specific T cells.

Experiments using autologous LCLs as stimulators of an EBV-specific T cell response showed that maximal responses were detected from PBMCs and whole blood after 16 hours. The assay was therefore shown to reliably and rapidly enumerate the functional EBV-specific immune response. The assay could be set up at the end of

a working day, incubated overnight, analysed and results communicated to clinicians the next day. In this way, decisions about reducing immunosuppressive therapy in patients with PTLD can be tailored by the clinician appropriately within 24 hours of the laboratory receiving a blood sample.

Experiments using autologous LCLs as stimulators of an EBV-specific T cell response also highlighted both variations in the magnitude of the response between healthy individuals, and also within individuals over time. This was particularly evident in two healthy individuals who were young (< 40 years) and who had acquired primary EBV infection less than 5 years prior to the start of monitoring. Compared to the two older healthy individuals (41 and 63 years respectively), the mean response was higher and wide fluctuations in the response were seen over time. This data is consistent with evidence that the number of functional EBV-specific T cells declines with advancing age (Khan *et al.*, 2004), a response that may be influenced by coinfection with another persistent herpes virus, CMV, which had been strongly implicated in immune senescence (reviewed by (Pawelec *et al.*, 2005). Several studies have shown virtual disappearance of initially expanded virus-specific CD8⁺ T cell clones following primary infection with EBV (Callan *et al.*, 1998a; Annels *et al.*, 2000; Callan *et al.*, 1998b) and HIV (Pantaleo *et al.*, 1997), with new dominant clones emerging as the main components of the memory EBV T cell response (Annels *et al.*, 2000). These studies, however, only used one time point to examine the memory response, 1-4 years after primary infection (Annels *et al.*, 2000; Callan *et al.*, 1998a). Therefore, a dynamic process may be occurring in which new T cell clones continuously emerge in response to cumulative episodes of intermittent EBV viral reactivation that occur in the initial period after primary infection, and

could explain the fluctuations seen over time. Despite variation in the magnitude of the EBV-specific immune response, the assay consistently detected a positive EBV immune response at all time points and in all individuals regardless of age and time from initial EBV infection.

Production of LCLs requires 3-4 weeks of *in vitro* culture before B cells from a PBMC sample are immortalized, and unfortunately are often difficult to generate from patient samples. The possibility of using LCLs matched at single HLA class I alleles was investigated as an alternative to autologous LCLs, but these cells often failed to stimulate a detectable EBV-specific T cell response. Detection rates were improved by summing the responses from a panel of LCLs matched on single alleles but this approach has the disadvantage of requiring many more PBMCs per individual. A practical solution to this may be to stimulate the responder PBMCs with a mixture of LCLs matched on single alleles. LCLs would need to be mixed immediately prior to the assay as growing a mix of LCLs *in vitro* would probably lead to outgrowth of the fastest growing cell line. This approach would require optimisation to determine the numbers of each different LCL that could be used in the mixture without overcrowding the culture, however results from the autologous LCL experiments showed that the EBV specific immune responses were relatively consistent using responder to stimulator ratios between 2:1 and 1:2. Another option is to use LCLs matched on multiple HLA alleles as stimulators. This was investigated using patient samples and results are presented in chapter 4.

HLA class I restricted EBV peptides were also investigated as an alternative source of EBV antigens. A practical advantage for using peptides as stimulators of an EBV-specific T cell response is that they can be stored at -80°C and used as required. In

contrast, EBV-transformed LCLs require at least 24 hours of *in vitro* culture prior to being used as CD8⁺ T cell stimulators.

Screening healthy individuals for EBV-specific T cells by stimulation with individual HLA-A*02 restricted peptides produced variable patterns of response. This may reflect age and time from primary EBV infection as discussed previously. Responses to known immunodominant peptides were often negative or failed to show a consistent positive response in all the individuals tested. Reasoning that combining peptides tailored to the HLA class I type of the responder cells should produce summation of individual responses, peptide pools were tested. The percentage of responding cells increased using a combination of peptides tailored to the HLA type of the responder cell and was able to detect a positive response in 5 of 6 individuals tested. Therefore, although the magnitude of the EBV-specific T cell response was less than with autologous LCLs, peptide pools could be used as an alternative source of EBV antigen in individuals with common HLA alleles.

One interesting finding with the peptide pools was that the positive response consistently failed to reach the sum of the individual peptide components. This was observed when individual peptides restricted through one HLA class I allele were combined in a peptide pool, and also when several peptide pools restricted through different alleles were combined. A possible explanation for the absence of complete summation of responses to individual peptides is competition for binding to class I molecules. Peptides presented by the same HLA class I allele compete for binding. This phenomenon is exploited for identifying peptides bound by HLA molecules and for quantifying binding affinities. Putative T cell epitopes are combined with a known HLA binding peptide and competition for class I binding assessed directly by

monitoring for reduction in binding of the labelled known class I binding peptide (Sette *et al.*, 1994; Ruppert *et al.*, 1993). Results from these studies have shown that the affinity of EBV peptide epitopes for binding to class I molecules range from 2.3-360.0nM (The Immune Epitope Database and Resource Analysis (IEDB), www.beta.immuneepitope.org). When peptides with differing affinities are bound and presented by the same HLA class I allele, those with high affinity will be preferentially bound and inhibit binding of the peptides with lower binding affinities. The impact on T cell recognition is reduction in responses to the lower affinity peptides because the amount of antigen is less.

Competitive inhibition of peptide binding may also account for the absence of complete summation of responses seen when combining multiple peptide pools restricted to different HLA allele. Although each class I allele has a distinctive peptide binding preference, known as the peptide binding motif, overlap in the repertoires of peptides bound by different allele has been described (Levitsky *et al.*, 2000; Livingston *et al.*, 1999; Threlkeld *et al.*, 1997). Class I allele are grouped into 'super-types' based on shared structural features in the peptide binding motif and thus similar peptide binding specificities (Sette and Sidney, 1999). An example pertinent to this study is the HLA-B*44 super-type comprising of a preference for peptides with negatively charged residues (D,E) at position 2 and hydrophobic residues at the C-terminus (DiBrino *et al.*, 1995; Harris *et al.*, 1993; Thorpe and Travers, 1994). It has been suggested that HLA-B*1801 also shares the same specificities as members of the HLA-B*44 super-motif group (Steven *et al.*, 1997). Therefore failure to summate the T cell response from the individual HLA-B*4402 and B*1801 peptide pools with a combination of both pools (**Figure 3.12**) and may reflect competitive inhibition.

Possible ways of reducing the effects of competitive inhibition include titrating each of the individual peptide components and using the minimum concentration of peptide required to produce a maximal T cell response. In the experiments presented here, all peptides were added at the same concentration (10µg/ml) and therefore the high affinity peptides are likely to be present in excess augmenting competitive inhibition within the pool.

The weak responses to stimulation with HLA class I restricted peptides may be improved by enhancing the function of APCs. A preliminary assessment of adding additional co-stimulation to cultures was undertaken using anti-CD28 antibodies, however no improvement was observed (data not shown). Alternative ways of maximizing antigen presentation that could be investigated include stimulating the PBMCs with EBV peptide-pulsed autologous PHA blasts (Bollard *et al.*, 2004) or dendritic cells (Redchenko and Rickinson, 1999; Santodonato *et al.*, 2003; Subklewe *et al.*, 1999; Subklewe *et al.*, 2005). However, the disadvantage with both strategies is that additional *in vitro* culture would be required resulting in a longer assay duration. As previously discussed such an approach is impractical in the context of PTLT, where the clinical effectiveness of the assay rests on how quickly results can be generated and communicated back to the clinicians responsible for reducing immunosuppression.

In conclusion, results presented in this chapter demonstrate that EBV-specific T cells can be rapidly and reliably enumerated from PBMCs and whole blood of healthy sero-positive individuals using autologous LCLs as stimulators. In addition, the assay fulfils many important criteria necessary for successful use in the clinical setting. Autologous LCLs from healthy individuals are relatively easy to establish *in vitro*,

and can be cryopreserved in liquid nitrogen until required. As liquid nitrogen storage is possible in most clinical laboratories, the additional cost of setting up an LCL is minimal. By definition, autologous LCLs present multiple EBV epitopes restricted by all HLA class I alleles recognised by the responder T cell. Therefore a positive EBV-specific response using autologous LCLs can be reliably demonstrated from 4 samples (autologous LCL (test), HLA-mismatched LCL (negative control), PHA stimulated PBMCs (positive control) and unstimulated PBMCs (negative control) requiring only 4×10^6 PBMCs. In contrast, use of LCLs matched at single HLA class I alleles involves testing responses to a panel of LCLs to accommodate the complete HLA-A, B and Cw type of the responder cells requiring large amounts of blood which is not practical in the clinical setting. A combination of multiple peptide pools tailored to the individual's HLA class I type was shown to stimulate a positive EBV-specific T cell response in the majority of individuals tested. However an assay based on use of peptide pools for the general population is currently hindered by lack of knowledge of the epitopes presented by less common HLA class I alleles. Using flow cytometry as the platform for analysis has the advantage of using equipment that is already available to most clinical immunology departments and a technique familiar to laboratory technicians so initial start-up costs would be low. Enumeration of IFN- γ producing CD8⁺ T cells by ICS and flow cytometry therefore represents a reliable method for detecting functional EBV specific T cell immunity in a manner which is highly amenable to clinical applications.

Chapter 4

Development of a rapid immunoassay to measure EBV-specific T cell responses: Patients with EBV- driven malignancies

4.1 INTRODUCTION

In the previous chapter, staining for intracellular IFN- γ production in CD8⁺ T cells from healthy individuals, stimulated overnight by autologous EBV-transformed LCLs or HLA-restricted peptide pools was shown to be a reliable and rapid means of enumerating an EBV-specific T cell response. The work described in this chapter assesses the clinical value of this assay by investigating its use to measure EBV-specific T cell responses in patients with two EBV-driven malignancies - PTLD and EBV-positive HD.

Reduction of immunosuppression (RIS) is widely used as the initial strategy in the treatment of patients with PTLD. This allows the patient to recover effective immunity to EBV and the functional EBV-specific T cells control EBV-driven cellular proliferation. Transplant patients, however, require immunosuppression to control detrimental T cell alloreactivity that causes rejection of solid organ allografts. Depending on the graft and host conditioning of haematopoietic patients receiving stem cell or bone marrow transplants, immunosuppression may also be required to prevent GVHD. Any withdrawal of immunosuppression risks compromising suppression of T cell alloreactivity and an approach that monitors stepwise withdrawal of immunosuppression so as to detect the recovery of EBV-specific cellular immune responses before damaging allograft reactivity would be of significant clinical benefit.

Current methods used to monitor recovery of the cellular immune response in patients with PTLD include quantifying activated CD8⁺ effector cells using immunophenotyping of surface activation markers by flow cytometry (Amlot *et al.*,

1996; Rees *et al.*, 1998; Amlot *et al.*, 2007). Patient samples are monitored from the diagnosis of PTLD throughout RIS to detect an increase in cell surface expression of lymphocyte activation markers with MHC Class II being the most informative. An absolute three-fold rise in the number of CD8⁺ T cells expressing MHC class II to achieve at least $0.3 \times 10^9/L$ is used to demonstrate sufficient recovery of the cellular immune response and adequate reduction in the levels of immunosuppression necessary for the treatment of PTLD (Amlot *et al.*, 2007). Although it may take several weeks after the appearance of these activated CD8⁺ T cells before a clinical response to the PTLD occurs, further reductions in immunosuppression are not required. The main disadvantage with this method is that it does not provide information regarding the EBV antigen specificity of the CD8⁺ effector cells. Immune responses to other co-existing infections may produce misleading results.

Another parameter that can be used to identify patients at high risk of developing PTLD is the EBV load. There are a variety of methods for measuring EBV load (described **section 1.4.3**) but at present no standardised protocols exist. As might be expected, some studies have shown that EBV load can predict the development of PTLD and that viral load falls in patients that respond to immunosuppression reduction (Lee *et al.*, 2005; McDiarmid *et al.*, 1998). However results are ambiguous as other studies show no value in measuring EBV load (Scheenstra *et al.*, 2004; Vajro *et al.*, 2000). Indeed treatment can result in a transient rise of viral load in blood plasma probably due to release of antigen by immune-mediated lysis of EBV-infected B cells (Savoldo *et al.*, 2006).

Use of the immunoassay described in Chapter 3 to quantify EBV-specific CD8⁺ effector T cells in EBV-positive PTLD patients as immunosuppression is reduced

should represent a better measure of response to treatment because the control of EBV in resting B cells and of EBV-positive PTLN is directly related to the immune response to EBV.

Another malignancy that is associated with EBV is HD. Approximately 40-50% of tumours are EBV-positive. The role of EBV in the aetiology of HD and the efficacy of EBV specific immunity in patients with HD is not well understood. EBV positive HD is more prevalent among older adults (>45 years) and young children. Some studies indicate that EBV-positive HD has a poorer prognosis (Keegan *et al.*, 2005b; Enblad *et al.*, 1999; Jarrett *et al.*, 2005). These observations may reflect compromised EBV immunity due to the immune naïve status of children and decline of the immune response in older patients.

A paradox in the pathogenesis of EBV-positive HD is that despite an effective cellular immune response to EBV after primary infection, patients subsequently fail to control proliferation of the EBV-infected malignant R-S cells in HD. Evidence suggests that immune avoidance strategies are employed by the virus, leading to persistence in the R-S cells. One strategy is down regulated expression of the EBNA 3A, 3B and 3C antigens by the virus so that R-S cells express only EBNA1, LMP1 and LMP2 known as latency pattern II. Studies suggest peptide epitopes from these antigens presented by HLA molecules stimulate relatively weak EBV specific T cell responses (Chapman *et al.*, 2001; Frisan *et al.*, 1995; Roskrow *et al.*, 1998). Cytotoxic T cells specific for EBNA-1 have not been detected and studies indicated this is because the antigen possesses a Glycine–Alanine repeat sequence that inhibits proteolytic degradation and therefore generation of peptide T cell epitopes by the proteasome (Blake *et al.*, 1997; Levitskaya *et al.*, 1995; Levitskaya *et al.*, 1997). However a more

recent study questions this conclusion because IFN- γ producing CD8⁺ T cells specific for EBNA-1 have now been detected (Lee *et al.*, 2004). Despite exploitation of immune avoidance strategies by EBV in HD, two pilot studies involving adoptive transfer of EBV specific T cells to control HD have had some success, as discussed in **section 1.5.5.3** of the Introductory Chapter (Bollard *et al.*, 2004; Lucas *et al.*, 2004). Both studies used EBV-specific CTLs that had been generated from *in vitro* stimulation with LCLs and were therefore specific for all EBV antigens. However, tetramer and functional analyses demonstrated that it was the T cells specific for LMP2 that expanded *in vivo* and entered the tumour (Bollard *et al.*, 2004). More information on the functional phenotype and antigen specificity of EBV specific T cells in HD patients is evidently required. These details could be deduced using the immunoassay described in Chapter 3. In addition, the assay could also be utilized to monitor responses to novel immune-based therapies for HD that aim to augment protective T cell responses.

Another factor contributing to persistence of EBV-infected R-S cells in HD patients is that any EBV-specific T cells that penetrate the tumour tissue may be rendered anergic by the immunosuppressive environment within the malignant lymph nodes. R-S cells secrete IL-10 (Herbst *et al.*, 1996; Ohshima *et al.*, 1995), TGF- β (Hsu *et al.*, 1993) and the Thymus and Activation-Regulated Chemokine (TARC) (Peh *et al.*, 2001; van den *et al.*, 1999), and recruit regulatory T cells (Marshall *et al.*, 2004; Alvaro *et al.*, 2005). In addition, development of an EBV-specific immune response seems to be inhibited through EBI3, an EBV-induced cytokine homologous to the p40 subunit of IL-12 and highly expressed in R-S cells that antagonizes IL-12 (Niedobitek *et al.*, 2002). Studies need to be performed to determine if the CD4⁺ tumour

infiltrating lymphocytes (TILs) in EBV-positive HD are functional and contribute to anti tumour immunity or if they are counter-productive regulatory T cells that suppress other tumour reactive cells. This could be achieved using the assay described Chapter 3.

The aims of the work described in this chapter are twofold. First, measurement of IFN- γ production by EBV-specific T cells (after stimulation with LCLs and HLA class I-restricted pooled EBV peptides) in patients with PTLD was performed serially as immunosuppression was withdrawn. Results were correlated to clinical response and compared with the conventional immunophenotyping for surface activation marker expression by T cells that is currently used by clinical laboratories to monitor treatment efficacy.

Second, the assay was used to measure the EBV-specific CD8⁺ and CD4⁺ T cell responses in a different EBV-associated tumour model. By using both TILs and PBMCs of patients with EBV-positive and EBV-negative HD it was possible to examine whether defects in anti-EBV immunity existed in the EBV-positive HD patients and could be responsible for ineffective tumour control.

4.2 RESULTS

4.2.1 Measurement of EBV-specific CD8⁺ T cell responses in patients with PTLD

4.2.1.1 Patient Characteristics

The characteristics of the 12 patients used in this study are listed in **Table 4.1**. All patients developed a PTLD after receiving a renal transplant. The male to female ratio was 2:1 and the average age at diagnosis was 57 years. Two patients presented within 1 year of transplantation, but most presented several years post transplant. Eight patients developed an EBV-positive PTLD (confirmed by the presence of EBER by in situ hybridization) with a mean time from transplant to diagnosis of 74.2 months. Seven of the eight EBV-positive PTLD patients had a clinical response to treatment with RIS (3 had a complete response, 4 had a partial response). All 4 patients who had a partial response and the one patient who had no response to RIS did not respond to further therapy and died of their PTLD or associated complications.

4 patients developed an EBV-negative PTLD with a mean time from transplant to diagnosis of 118.3 months. 3 patients were initially treated with reduction of immunosuppressive therapy and 1 patient with cytotoxic chemotherapy. Of the 3 patients that were treated with RIS, one had a complete clinical response to treatment, one had no response and was treated with chemotherapy and rituximab, and one was not evaluable as the lesion was removed surgically.

All clinical responses to treatment were measured by standard radiological imaging and defined according to the RECIST criteria (**Appendix 1.3**, (Therasse *et al.*, 2000)).

<i>Patient Code</i>	<i>Age at Tx (yrs)/ Sex</i>	<i>Pre-Tx EBV serostatus</i>	<i>Time from Tx to PTLT (months)</i>	<i>Site of Disease</i>	<i>Treatment Regime</i>	<i>Clinical Outcome</i>
EBV⁺ 1	57/M	Unknown	129	Skin	Surgery/RIS	CR
EBV⁺ 2	68/M	Negative	4	Generalised LN, BM Pleura	1. RIS 2. Rituximab	NR †
EBV⁺ 3	68/F	Unknown	95	Sigmoid colon	1. RIS 2. Rituximab	PR MR †
EBV⁺ 4	65/F	Unknown	11	Perirenal mass	1. RIS	CR
EBV⁺ 5	40/M	Negative	42	Tonsil	1. RIS/CTL	CR
EBV⁺ 6	68/M	Unknown	216	Skin	1. RIS/RT	PR †
EBV⁺ 7	59/M	Unknown	39	Pancreas	1. RIS 2. Chemo	PR NR †
EBV⁺ 8	63/M	Negative	58	Generalised LN	1. RIS 2. Chemo	PR †
EBV⁻ a	34/F	Unknown	150	Mesenteric/ para-aortic LN, Bowel	1. RIS 2. Chemo + Rituximab	NR CR
EBV⁻ b	59/M	Positive	80	Neck LN	1. RIS	CR
EBV⁻ c	65/F	Positive	100	Skin	1. RIS 2. Radiotherapy	NE
EBV⁻ d	40/M	Unknown	143	Tonsil, Neck and abdominal LN	1. RIS 2. Chemo	NR CR

Table 4.1: Characteristics of patients who developed a PTLT following renal transplantation

EBV status of tumour: EBV⁺ positive; EBV⁻ negative M, male; F, Female; LN, Lymphadenopathy; Chemo, Chemotherapy; RIS, Reduction in immunosuppression; CTL, adoptive cytotoxic T cell infusion; RT, Radiotherapy; BM, Bone marrow; CR, complete clinical response; PR, partial clinical response; MR, minor response; NR, no response; NE, not evaluable. † died.

4.2.1.2 Patient sample collection and EBV antigen stimulation conditions tested

The number of samples, mean cell number and viability of thawed cryopreserved cells obtained from each patient are shown in **Table 4.2**.

To measure the EBV-specific T cell responses in patients with PTLD, PBMCs from 12 patients were collected at diagnosis and at multiple time points during treatment and EBV specific T cell mediated immunity assessed using the intracellular cytokine assay optimized in Chapter 3. Autologous LCLs consistently stimulated the largest CD8⁺ T cell response in healthy individuals and this was re-assessed in the PTLD patient group. Wherever the numbers of PBMCs from patients permitted, pooled EBV peptides tailored to the patients HLA class I type and a panel of partially HLA-matched LCLs were evaluated along with autologous LCLs. For five patients autologous LCLs could not be generated, possibly due to their immunosuppressed status at the time of diagnosis. For these patients, only LCLs matched on single and multiple HLA class I alleles were tested (**Table 4.3**). At each time point, the EBV-specific T cell responses were compared to the percentage of MHC class II⁺ CD8⁺ T cells obtained from the lymphocyte activation immunophenotyping data performed as a routine clinical monitoring service for all PTLD patients in the Department of Clinical Immunology, Royal Free Hospital.

<i>Patient Code</i>	<i>Number of samples</i>	<i>Cell Number ($\times 10^6$) Mean (sd)</i>	<i>Viability (%) Mean (sd)</i>
EBV⁺ 1	3	17.3 (4.62)	97.3 (2.08)
EBV⁺ 2	1	20.0	96.0
EBV⁺ 3	6	20.5 (10.17)	97.8 (0.75)
EBV⁺ 4	9	10.9 (5.80)	95.7 (3.28)
EBV⁺ 5	8	6.3 (2.25)	96.9 (2.10)
EBV⁺ 6	5	7.6 (1.95)	90.8 (6.38)
EBV⁺ 7	2	4.5 (0.71)	89.5 (3.54)
EBV⁺ 8	2	10.5 (2.12)	92.5 (7.78)
EBV⁻ a	2	7.5 (3.53)	87.5 (10.6)
EBV⁻ b	3	2.3 (0.58)	92.6 (3.06)
EBV⁻ c	5	9.0 (1.58)	96.4 (1.82)
EBV⁻ d	4	2.2 (0.50)	94.5 (2.08)
Total Mean		9.22	94

Table 4.2: Patient samples

<i>Patient Code</i>	<i>Patient HLA-A, B, C type</i>	<i>Autologous LCL</i>	<i>HLA-matched LCL (Number)</i>	<i>Peptide Pool (Number of peptides)</i>
EBV⁺ 1	A*0201, A*1101 B*4402, B*2705 Cw*012, Cw*0501	N	Y A*0201 single (2) A*1101 single (1) B*4402 single (2) B*2705 single (1) A*1101+B*4402 (1) A*1101+B*2705 (2) A*0201+B*2705 (1)	Y (23)
EBV⁺ 2	A*0301 B*1402, B*5101 Cw*0102, Cw*0802	N	Y A*0301 single (1) B*5101 single (1) A*0301+Cw*0802 (1) A*0301+B*5101 (1) B*5101+Cw*0102 (1)	Y (1)
EBV⁺ 3	A*0101, A*0301 B*5701, B*3501/7/8 Cw*04, Cw*0602	N	Y A*0101 single (2) A*0301 single (2) Cw*04 single (2) Cw*0602 single (2) B*3501+Cw*0401 (2) A*0301+B*3501+Cw*0401 A*0101+B*5701+Cw*0602	Y (4)
EBV⁺ 4	A*0201, A*2402 B*5101 Cw*0202, Cw*1502	Y	N	Y (19)
EBV⁺ 5	A*0101, A*0301 B*0702, B*0801 Cw*0701, Cw*0702	Y	Y A*0101 single (1) A*0301 single (1) B*0702 single (1)	Y (9)
EBV⁺ 6	A*0101, A*0201 B*0801, B*4001 Cw*0304, Cw*0701	Y	N	Y (21)
EBV⁺ 7	A*0101, A*2402 B*5501, B*3501 Cw*0303, Cw*0401	Y	N	Y (6)
EBV⁺ 8	A*0101, A*2402 B*0702, B*1801 Cw*0701, Cw*0702	N	Y A*0101 single (1) A*2402 single (1)	N
EBV⁻ a	A*0201, A*0301/3, B*1801, B*0702	Y	Y A*0301 single (2) A*0201 single (2) A*0301+B*1801 (1)	Y (20)
EBV⁻ b	A*0101 B*0801 Cw*0701	Y	N	N
EBV⁻ c	A*0101, A*0201 B*4402, B*0801 Cw*0501, Cw*0702	N	Y A*0201 single (1) B*4402 single (1) A*0101+A*0201+B*4402 A*0101+B*0801+Cw*0702	Y (24)
EBV⁻ d	A*6801, A*6802 B*4402, B*1402 Cw*0704, Cw*0802	Y	Y B*4402+B*1402 (1)	N

Table 4.3: Sources of EBV antigen used to stimulate an EBV-specific CD8⁺ T cell response in each PTLD patient

PBMCs from 12 PTLD patients were stimulated with autologous LCLs, HLA-matched LCLs or HLA-restricted peptide pools as indicated (Y or N). Where autologous LCLs could not be generated, a panel of single HLA-matched LCLs e.g. A*0201 single = a LCL matched on A*0201 allele alone) and LCLs matched on at least two HLA-alleles (e.g. A*2402+B*0702+Cw*0701) were used. The number in brackets after each line refers to the number of different LCLs matched for the allele that were tested. The number of peptides in each pool is shown in brackets.

4.2.1.3 Measurement of EBV-specific CD8⁺ T cell responses in PTLD patients with EBV-positive tumours during RIS

Figure 4.1 shows the serial data for the IFN- γ producing CD8⁺ T cell responses and the RIS strategy, in conjunction with creatinine measurements in three EBV-positive PTLD patients that each had more than 5 serial samples available for study.

Panel a represents data from EBV⁺ 4 who presented with a peri-renal mass 11 months after renal transplantation. Histology demonstrated a diffuse, large B cell lymphoma which was EBER positive. She was treated with RIS alone and monitored for an increase in activated lymphocyte subsets. **Figure 4.1** shows that by 4 months there had been a three-fold increase in the absolute number of MHC Class II⁺CD8⁺ T cells and further RIS was stopped. EBV⁺ 4 went on to achieve a complete clinical remission by standard imaging criteria. RIS in this patient was complicated by a steady increase in the baseline creatinine measurements. A biopsy at 6 months confirmed graft rejection which was treated with an increase in the dose of immunosuppressive drugs.

Panel b represents data from EBV⁺ 3 who presented with a mass within the sigmoid colon and abdominal lymphadenopathy 7.9 years after renal transplantation. The histology of the tumour also confirmed a diffuse large B cell lymphoma which was

EBER positive. EBV⁺ 3 was treated with RIS and monitored by immunophenotyping for lymphocyte activation markers. By 3 months the absolute number of MHC Class II⁺CD8⁺ T cells had increased by three-fold and further RIS was halted. EBV⁺ 3 had an initial partial response to RIS, however recurrent diarrhoea and rectal blood loss, suggestive of tumour recurrence at 12 months after diagnosis led to further RIS. Despite withdrawal of all immunosuppression, persistent disease in the rectum and colon required second-line treatment with rituximab. EBV⁺ 3 had a minor response to rituximab at the abdominal lymph nodes but subsequently died from cachexia and intractable diarrhoea.

Panel c represents data from EBV⁺ 5 who was EBV sero-negative at the time of renal transplant and developed a primary EBV illness with encephalitis and a tonsillar mass 42 months after renal transplantation. A biopsy from the tonsillar mass confirmed an EBV-positive PTLN by EBER staining and the EBV viral copy number within the CSF was high. EBV⁺ 5 was treated with RIS and 4 infusions of HLA-matched allogeneic EBV-specific T cells, and was monitored by both immunophenotyping and serial EBV viral copy numbers from serum samples. By 3 months a three-fold increase in the absolute number of MHC Class II⁺CD8⁺ T cells had occurred and further RIS was stopped. Radiological imaging confirmed a complete clinical response to treatment.

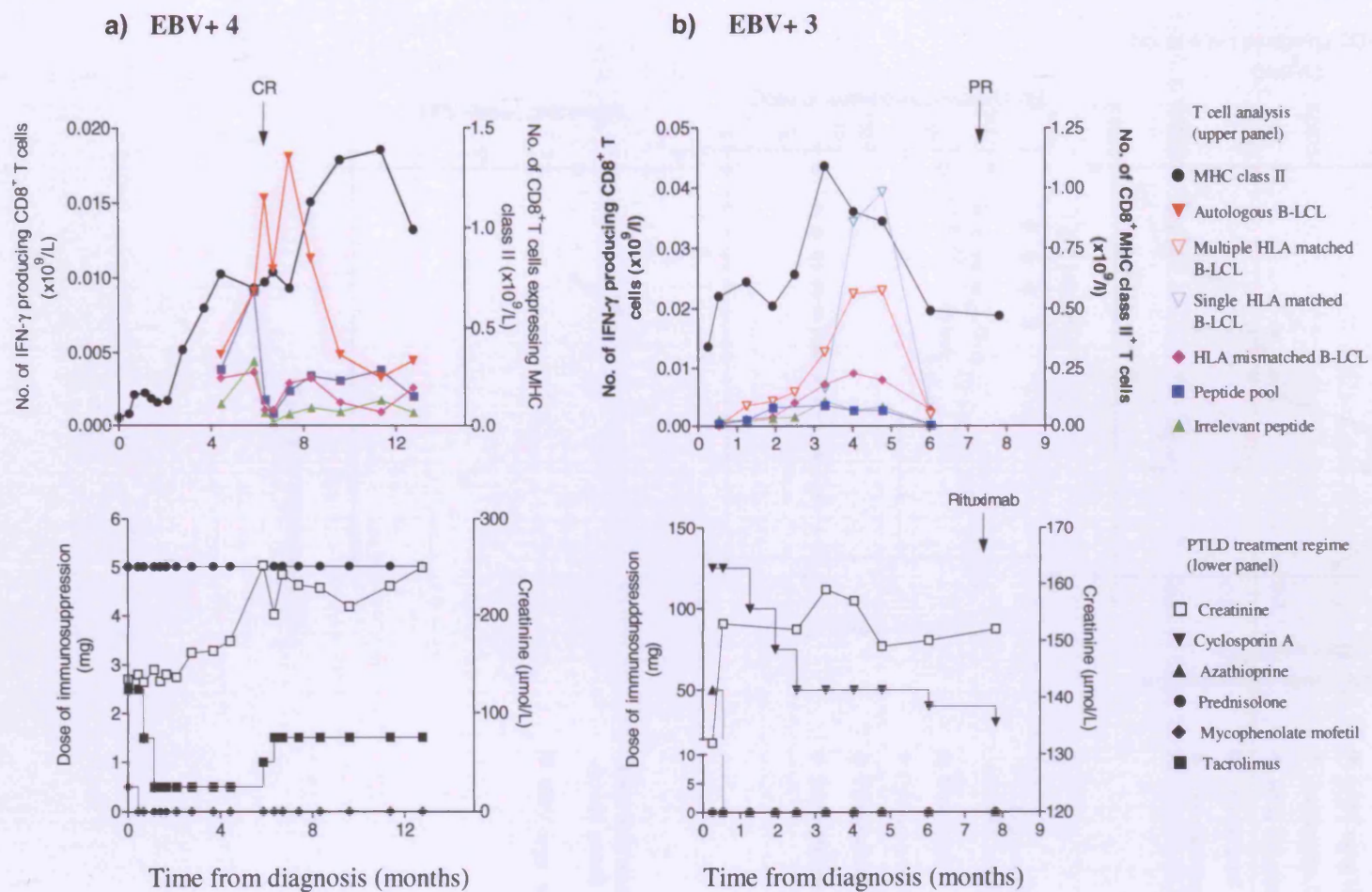
Figure 4.1 demonstrates that an increase of EBV-specific IFN- γ -producing CD8⁺ T cells accompanied clinical response to treatment. All eight EBV positive PTLN patients were treated with RIS. 3 patients (EBV⁺ 1, (**Appendix 1.4**) EBV⁺ 4 and EBV⁺ 5) had a complete response to RIS and demonstrated a rise in EBV-specific CD8⁺ T cells as immunosuppression was reduced. 4 patients (EBV⁺ 3, EBV⁺ 6, EBV⁺ 7 and EBV⁺ 8 (**Appendix 1.4**) had a partial response to treatment. Although

only limited time points were available, the number of EBV-specific CD8⁺ T cells in all four patients was high or rising after treatment with RIS. In two patients (EBV⁺4 and EBV⁺5) complete clinical response was associated with a subsequent decline in EBV-specific immunity; a response appropriate to the effective removal of EBV antigen. A decline in EBV-specific T cells was also observed in EBV⁺3. Although this patient had a partial response to RIS, she subsequently had a clinical relapse requiring further treatment, suggesting that a fall in EBV immunity prior to achieving a complete clinical response may predict relapse. EBV⁺2 (**Appendix 1.4**) was treated with RIS followed by rituximab and cytotoxic chemotherapy for a diffuse large B cell EBV positive PTLT tumour. The patient died on treatment secondary to neutropenic sepsis before any clinical response was seen, however EBV-specific CD8⁺ T cells were high after immunosuppression had been withdrawn, suggesting that functional EBV specific immunity was emerging.

The emergence of functional EBV-specific T cells was observed in the context of several different immunosuppressive regimes and occurred at different levels of reduction for each patient. A rise in EBV immunity occurred with an 80% RIS compared to pre-treatment levels of immunosuppression for EBV⁺4, 60% for EBV⁺3 and 10% for EBV⁺5 in combination with infused allogeneic EBV-specific T cells.

In addition to showing a rise with clinical response, the increase in EBV-specific CD8⁺ T cells also accompanied the rise in CD8⁺MHC Class II⁺ T cells. An exception was EBV⁺6 where a partial response to RIS was associated with an increase in EBV-specific CD8⁺ T cells but no change in the cell surface expression of MHC class II. This patient was also unusual in presenting with late-onset EBV-positive PTLT, 18 years after transplantation.

In two patients (EBV'3, EBV'4) the time course of the expansion of functional EBV-specific T cells was more restricted than for the increase in MHC class II expression, suggesting that the latter encompasses the EBV-specific response as well as other activation related processes that may include an increase in T regulatory cells. Although data on EBV viral load was available for only one patient (EBV' 5), **Figure 4.1** also demonstrates a relationship between the frequency of EBV-specific CD8⁺ T cells and EBV load. Patient EBV' 5 (**Panels C and D**) had a complete response to treatment with RIS and CTL therapy which accompanied a rise in the frequency of EBV-specific CD8⁺ T cells and a fall in the EBV viral copy number. The viral copy number at 2 months after diagnosis (0.0 genome/mL) is thought to be an erroneous result. EBV' 5 therefore provides an example where EBV load corresponds with response to treatment for PTLN (Smets *et al.*, 2002)



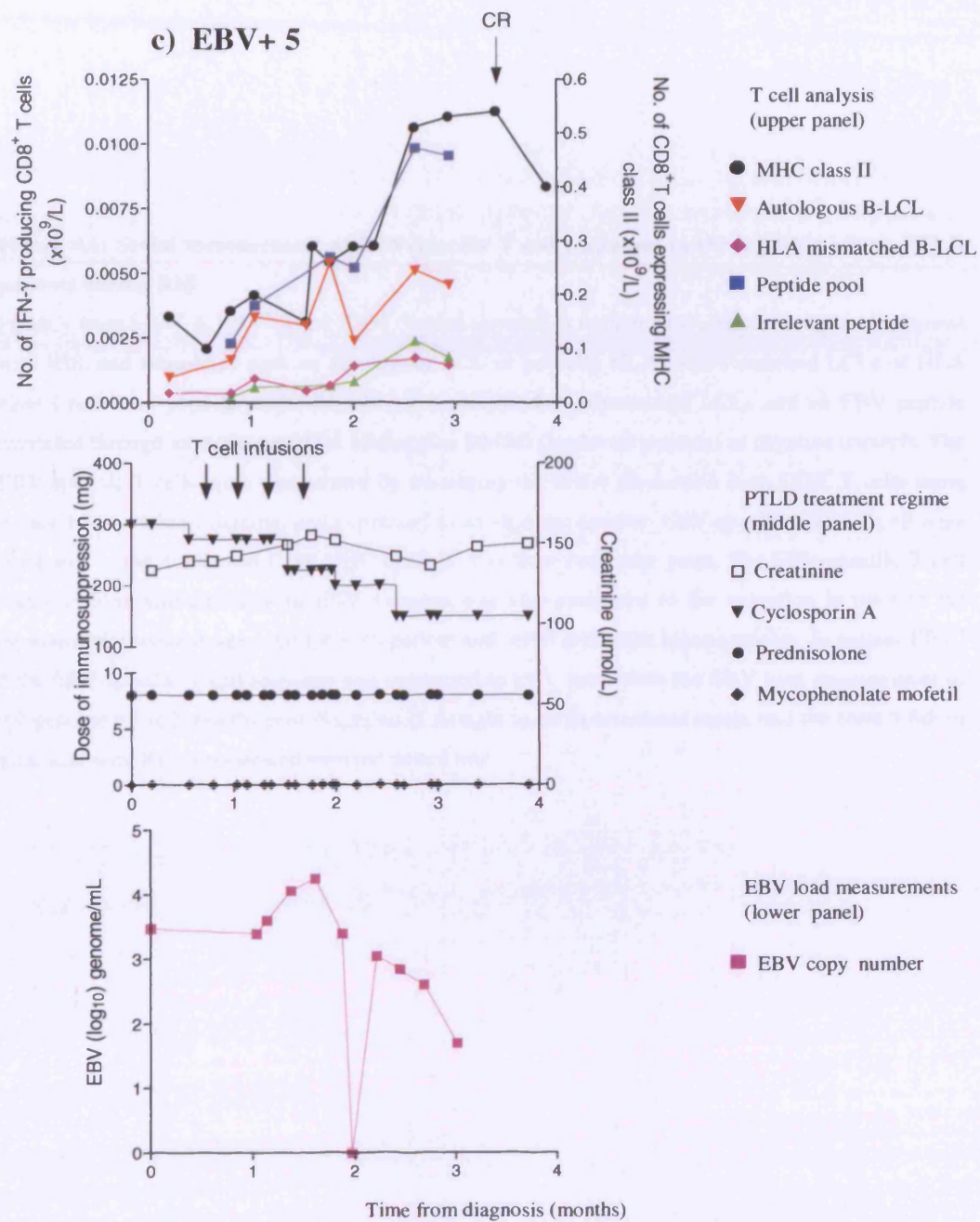


Figure 4.1: Serial measurement of EBV-specific T cell responses in three EBV-positive PTLD patients during RIS

PBMCs from EBV⁺ 4, EBV⁺ 3 and EBV⁺ 5 were sampled at regular time points throughout treatment with RIS, and stimulated with an autologous LCL or partially HLA class I matched LCLs or HLA class I restricted peptide pools. Results were compared to mismatched LCLs and an EBV peptide restricted through an irrelevant HLA alleles plus DMSO (Irrelevant peptide) as negative controls. The EBV-specific T cells were enumerated by measuring the IFN- γ production from CD8⁺ T cells using intracellular cytokine staining, and expressed as an absolute number. EBV-specific CD8⁺ T cell were compared to the number of CD8⁺MHC class II⁺ T cells at each time point. The EBV-specific T cell response from stimulation with EBV antigens was also compared to the reduction in dose of the immunosuppressive drugs used for each patient and serial creatinine measurements. In patient EBV⁺ 5 the EBV-specific T cell response was compared to EBV load. Note the EBV load measurement of 0.0 genome/ml at 2 months post diagnosis is thought to be an erroneous result, and the correct fall in viral load with RIS is suggested with the dotted line.

4.2.1.4 *Assessment of EBV-specific CD8⁺ T cell responses in PTLD patients with EBV-negative tumours during RIS*

EBV-negative PTLDs usually occur late after transplantation and are often more aggressive resulting in a poor prognosis compared to EBV-positive PTLD (Leblond *et al.*, 1998). The pathogenesis for EBV-negative PTLDs remains unclear; some studies have suggested that they represent sporadic lymphomas that have occurred in immunosuppressed patients and should not be considered under the PTLD classification umbrella (Dotti *et al.*, 2000; Sivaraman and Lye, 2001). Other studies, however, have suggested that EBV was initially involved in the oncogenesis of EBV-negative PTLD tumours but loss of the EBV antigen subsequently occurred through a series of mutations resulting in viral-independent cell proliferation. Whether this event was driven by selection pressure to avoid the vigorous cellular immune response to EBV is unclear. Support for this proposed ‘hit and run’ oncogenesis theory (Ambinder, 2000) includes evidence that some clones of the EBV-associated Burkitt’s lymphoma-derived cell line, Akata, (Shimizu *et al.*, 1994) and the nasopharyngeal carcinoma cell line, NPC-C666, (Chen *et al.*, 1999) are capable of losing their viral episomes when grown in culture. In addition, a recent series of non-endemic Burkitt’s lymphomas showed that in some cases where EBV could not be detected by standard molecular techniques, partial fragments of EBV genome were evident (Razzouk *et al.*, 1996).

Despite loss of EBV antigen, a small percent of EBV-negative PTLD patients will respond to RIS (Nelson *et al.*, 2000). The basis for a clinical response to RIS is unknown but thought to involve the emergence of anti-tumour reactivity that is not mediated by EBV specific T cells. EBV immunity is unlikely to change during RIS and therefore this cohort of patients was included as a negative control.

Figure 4.2 shows the serial data from two EBV-negative PTLT patients with more than 5 serial samples who were treated with RIS. EBV⁻ b presented with cervical lymphadenopathy 6.6 years after renal transplantation. Histology showed an anaplastic large cell lymphoma which was negative for EBV by EBER staining. He was treated with RIS alone and went on to have a complete clinical response. EBV⁻ c presented 8.3 years after renal transplantation with multiple skin lesions involving the scalp. Histology showed a diffuse large B cell lymphoma which was negative for EBV markers, and she was treated with both RIS and localized radiotherapy to the scalp. To date she has achieved a partial clinical response.

In all four EBV-negative PTLT patients, (**Figure 4.2** and **Appendix 1.4**) there was no association between the level of EBV-specific IFN- γ producing CD8⁺ T cells and clinical response to treatment. This finding was consistent among the patients treated with RIS (EBV⁻ b and EBV⁻ c) and those treated with cytotoxic chemotherapy (EBV⁻ a and EBV⁻ d **Appendix 1.4**), suggesting that EBV immunity is unchanged by either therapeutic approach. The frequencies of MHC class II⁺ CD8⁺ T cells also did not show consistent change during RIS for all four patients.

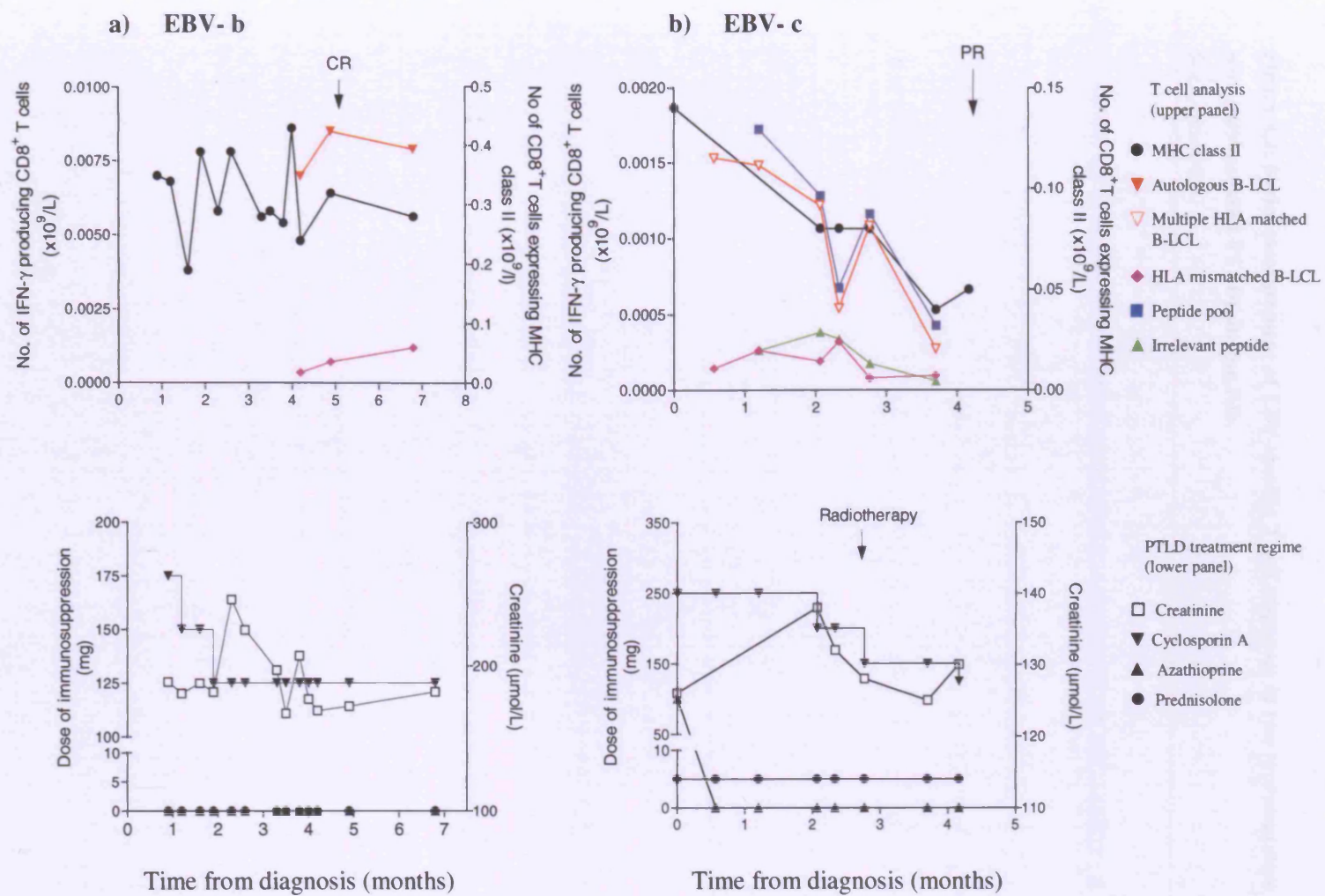


Figure 4.2: Serial measurement of EBV-specific T cell responses in two representative patients with EBV-negative PTLDs during RIS

See Legend 4.1

4.2.1.5 Comparison of peak EBV-specific T cell responses in patients with PTLD measured after stimulation with LCLs or HLA class I restricted peptide pools

A comparison of detection and size of the responses promoted after stimulation with autologous LCLs, partially HLA class I matched LCLs or peptide epitope pools was performed for 11 patients with PTLD. For each patient, the sample screened at the peak of functional EBV immunity was selected for study and the magnitude of the responses obtained by stimulation with each EBV antigen source compared. EBV⁺ 2 was not included as only one sample was available for assessment.

Figure 4.3 compares the magnitude of the response measured when functional EBV immunity was at its peak after stimulation with autologous LCLs, partially matched LCLs and HLA-restricted peptide pools in EBV-positive PTLD patients (**Panel A**) and EBV-negative PTLD patients (**Panel B**). Regardless of the EBV status of their PTLD tumours, IFN- γ producing CD8⁺ T cells specific for EBV were detectable in all patients after stimulation with at least one of the antigen sources tested. This result is expected given their history of previous EBV infection. Six patients had autologous LCLs available and in all cases these cells stimulated a positive EBV-specific T cell response. This made autologous LCLs the most reliable source of antigen to use for detection of EBV immunity and is consistent with results presented in Chapter 3 for studies with healthy individuals.

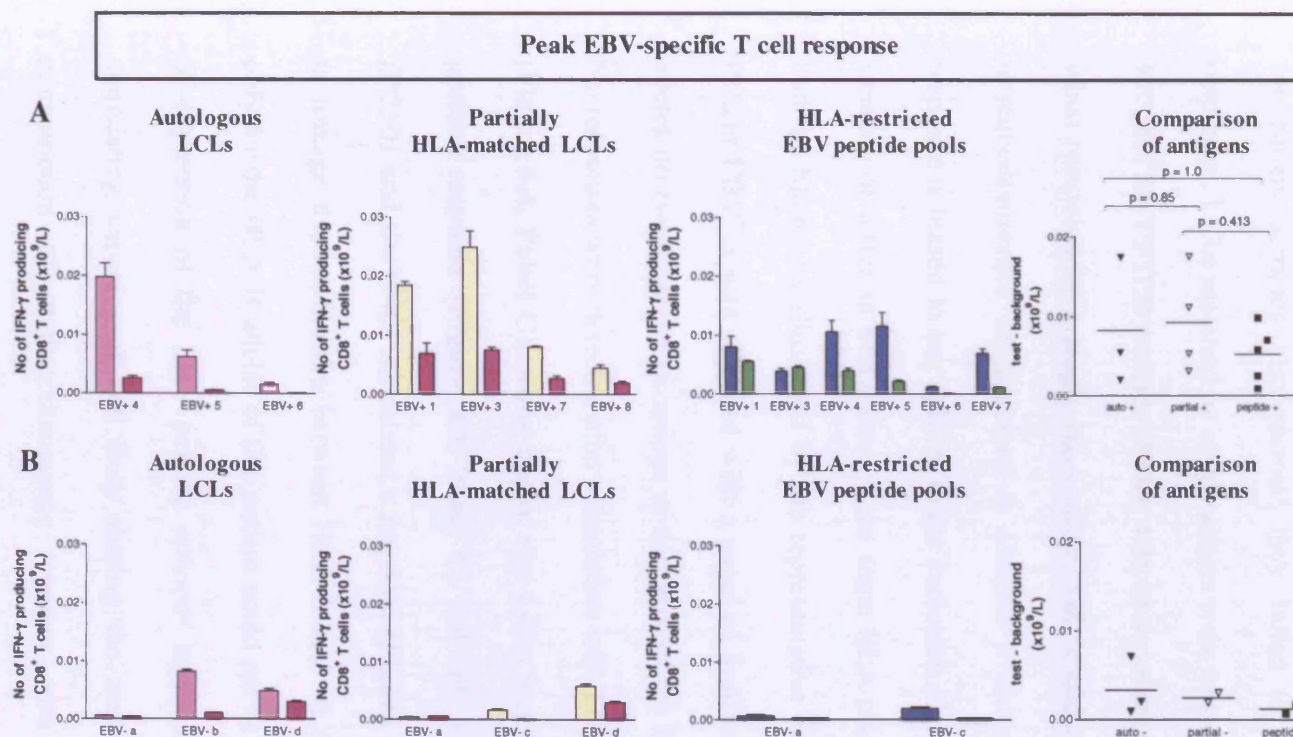


Figure 4.3: Comparison of peak EBV-specific T cell responses on stimulation with different EBV antigens in patients with PTLD

Results shown are those obtained at the peak of the EBV response for each patient. PBMC samples from EBV-positive (**Panel A**) and EBV-negative (**Panel B**) PTLD patients were stimulated with autologous LCLs (pink), partially matched LCLs (yellow), mismatched LCLs (magenta), HLA class I-restricted peptide pools tailored to patient HLA type (blue) or irrelevant peptide (green). EBV-specific T cells were quantified by measuring production of IFN- γ by CD8 $^{+}$ T cells using intracellular cytokine staining and expressed as an absolute number using the lymphocyte count. The bars and error bars represent the mean and sd respectively. The graphs shown far right of **Panels A** and **B** present the magnitude of the EBV-specific T cell responses after stimulation with each antigen source calculated from the test sample-relevant negative control (mismatched LCL or irrelevant peptide) to give increase above background. Statistical analysis to compare the magnitude of the positive responses generated after stimulation with each antigen source was performed using a Mann-Whitney U test.

Unfortunately, attempts to generate autologous LCLs are not always successful. Use of LCLs matched at a single HLA class I allele as an alternative source of stimulator cells was investigated for healthy individuals but found to be unreliable (see **Chapter 3 section 1.2**). Assessment of single HLA class I allele matched LCLs for patient samples also showed they failed to stimulate consistent positive responses. LCLs matched on single alleles were tested as alternative source of EBV antigens for 7 PTLN patients (**Table 4.3**). In some cases, no responses were detected when restricted by a single HLA allele. This could be due to the phenomenon of immunodominance as discussed in Chapter 3 section 1.2 whereby the immune response is biased to one epitope to the exclusion of others. Variation in stimulating capacity of different LCLs sharing the same HLA class I allele was also noted and both problems are illustrated by the representative example shown in **Figure 4.4**. Patient EBV⁺ 3 was screened with a panel of 8 different LCLs matched on single alleles on two separate occasions at the peak of the EBV-specific T cell response. No responses were detected after stimulation with LCLs matched at HLA-Cw*0602 (**Figure 4.4, Panel C**). Only one of two LCLs matched for Cw*0401 produced a positive response (**Figure 4.4, Panel C**) and only one of two LCLs matched at B*3501 and Cw*0401 stimulated a response (**Figure 4.4, Panel B**). Note that due to linkage disequilibrium between HLA-B and HLA-Cw alleles, LCLs matched only for the HLA-B alleles of this patient could not be obtained. Variation in levels of expression of the EBV peptide epitopes may account for differences in the stimulating capacity of cell lines sharing the same HLA class I allele. The phenomenon of immunodominance together with unpredictable variation in stimulating capacity of cell lines matched only at single HLA alleles would necessitate screening of several different LCLs for each patient to ensure all positive

responses are detected. However this would require testing a large number of PBMCs which cannot realistically be obtained from patients for routine analysis. It may be possible to circumvent these problems by using LCLs matched on multiple alleles or pools of peptide epitopes tailored to patient HLA type, so their use as alternative stimulators to autologous LCLs was investigated.

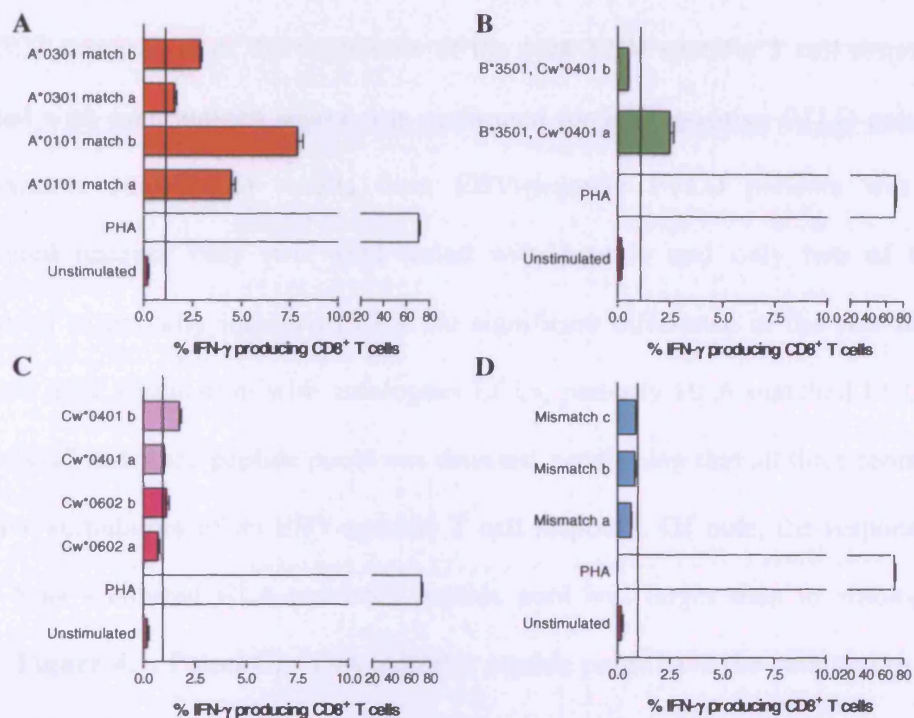


Figure 4.4: Representative example from patient EBV⁺3 of EBV-specific T cell responses after stimulation with a panel of single HLA class I allele matched LCLs

PBMCs from EBV⁺3 (HLA-A*0101, A*0301 B*5701, B*3501 Cw*04, Cw*0602) were stimulated with a panel of LCLs matched on HLA-A alleles alone (**Panel A**), both HLA-B and HLA-Cw alleles (**Panel B**) or HLA-Cw alleles alone (**Panel C**). Background stimulation was calculated from the mean value obtained with 3 different mismatched LCLs (**Panel D**) and is represented by black vertical lines in all panels. Data from this experiment is representative of results from two consecutive samples collected at the height of the EBV-specific T cell response (4 and 4.8 months post PTLD diagnosis). EBV-specific T cells were quantified by measuring IFN-γ production by CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells. The bar and error bar values represent the mean and sd values respectively.

Figure 4.3 shows positive EBV-specific T cell responses were detected in 6 of the 7 patients stimulated with LCLs matched on 2 or more HLA class I alleles and 7 out of 8 patients stimulated with HLA class I restricted EBV peptide pools. EBV⁺ 1 did not respond to an LCL matched at two alleles (A*0301 and B*1801) despite a positive response to autologous LCLs and EBV⁺ 3 did not respond to a peptide pool containing 4 epitopes despite a positive response to a partially matched LCL. A statistical comparison of the magnitude of the peak EBV specific T cell responses detected with each antigen source was performed for EBV-positive PTLD patients. Comparative analysis of results from EBV-negative PTLD patients was not performed because only two were tested with peptide and only two of three responded to partially matched LCLs. No significant difference in the size of the response after stimulation with autologous LCLs, partially HLA-matched LCLs or HLA class I restricted peptide pools was detected, confirming that all three represent effective stimulators of an EBV-specific T cell response. Of note, the response of EBV⁺ 5 to a tailored HLA-restricted peptide pool was larger than to autologous LCLs (**Figure 4.1, Panel C**). This indicates peptide pools have the potential to be a better source of antigen than autologous LCLs. All nine peptides in the pool for EBV⁺ 5 were immunodominant EBV antigens including the HLA-B*0801 restricted epitope **RAKFKQLL** from the immediate early lytic EBV antigen BZLF 1 that produces strong EBV-specific T cell responses in healthy individuals (Pudney *et al.*, 2005). The concentration of these immunodominant peptides in the pool may exceed that found on the surface of LCLs (Hill *et al.*, 1995) and account for why the response to a peptide pool can be greater than that to autologous LCLs. These results indicate that both partially matched HLA class I LCLs and peptide pools could be practical alternatives to autologous LCLs as a source of EBV antigen.

However, neither showed the absolute reliability of autologous LCLs - one false negative result was obtained with both antigen sources. LCLs need to be selected that are matched for the maximum number of HLA class I alleles. The negative result with peptides (EBV⁺3) was obtained with the pool comprising the smallest number of peptides (only 4) and therefore reliability is likely to improve as our knowledge of T cell epitopes grows.

4.2.1.6 Comparison of the magnitude of the EBV immune response at initiation of RIS with the maximum response detected

Comparison of the magnitude of the EBV response at initiation of RIS with the maximum response point was performed for EBV-positive and EBV-negative PTLD patients. Samples close to the start of RIS were not available for patients EBV⁺2, EBV⁺4, EBV⁺6, EBV⁺7 and EBV^b, precluding their inclusion in this analysis. Results for 4 EBV-positive and 3 EBV-negative patients are shown in **Figure 4.5**. There was no statistically significant difference in magnitude of the EBV response at the start of RIS between EBV-positive and EBV-negative PTLD patients ($p=0.4$) indicating that susceptibility to EBV-positive PTLD does not result from greater compromise of EBV immunity by immunosuppression in those patients. There was no statistical difference between the initial and peak mean responses for the EBV-negative PTLD patients ($p=0.4$) reflecting the absence of change in functional EBV immunity during RIS for EBV-negative PTLD patients (**Figure 4.2**). Comparison of the initial with the maximum magnitude of the EBV immune response identified a borderline statistically significant difference for the EBV-positive PTLD patients ($p=0.057$), with 2 patients (EBV⁺3 (175-fold increase) and EBV⁺5 (25-fold increase)) showing a 3-fold or greater increment in the number of EBV-specific CD8⁺ T cells. Patients EBV⁺4 and EBV⁺6 were not included in the analysis because

samples at early time points were not available. However, the serial measurements presented in **Figure 4.1a** and **Appendix 1.4** respectively shows these patients had impressive rises in EBV immunity. An absolute increase of 0.0023×10^9 IFN- γ producing CD8⁺ T cells at the first data point collected 4 months after RIS had begun, rising to a peak of 0.017×10^9 IFN- γ producing CD8⁺ T cells, representing a 7 fold increase was noted for EBV⁺4, and an absolute increase of 0.0003×10^9 IFN- γ producing CD8⁺ T cells at the first data point collected 6 months after RIS had begun rising to a maximum of 0.00199×10^9 IFN- γ producing CD8⁺ T cells, representing a 6 fold increase for EBV⁺6. These patients were therefore likely to have had low EBV immune responses at the start of RIS. If this is assumed, their inclusion in the analysis would have driven the p value comparing EBV immunity in EBV-positive PTLN patients at commencement of RIS with the maximum response to significance ($p=0.0152$).

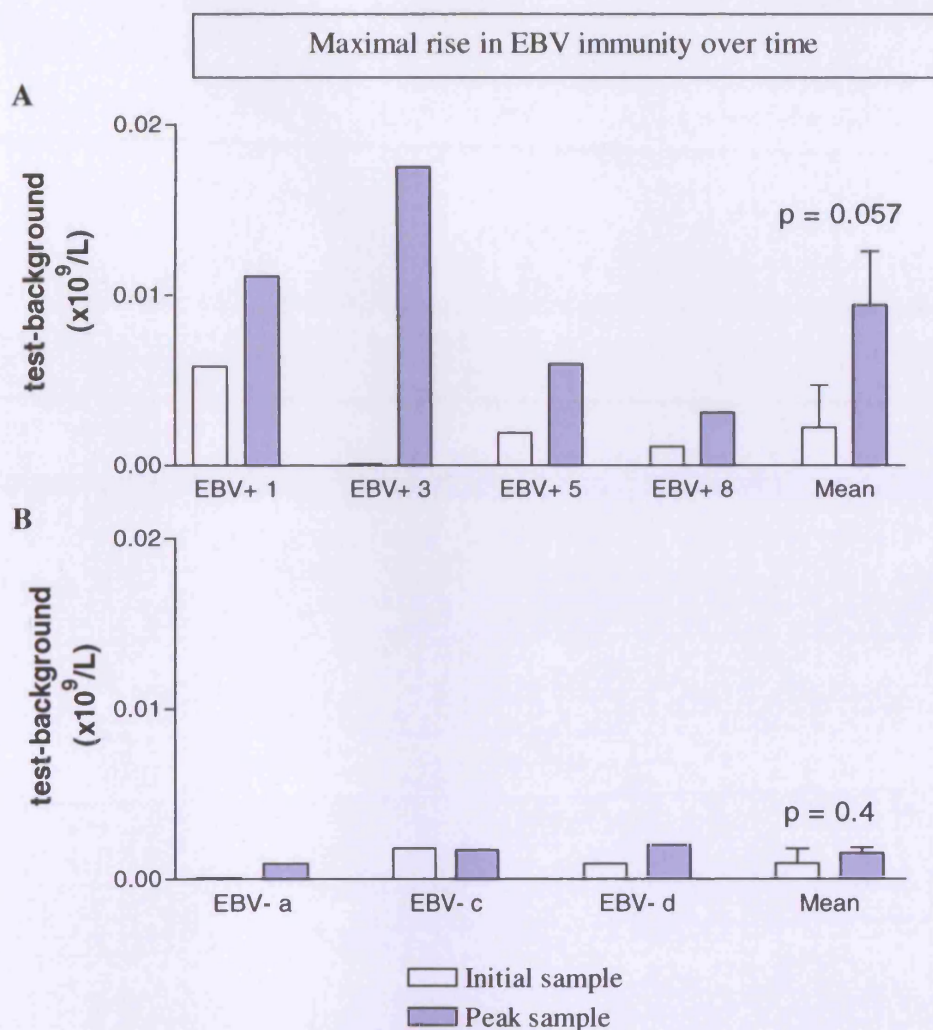


Figure 4.5: Magnitude of the EBV immune response at initiation of RIS compared to the maximum response detected

The magnitudes of the initial (white) and peak (blue) EBV-specific T cell responses for both EBV-positive (A) and EBV-negative PTLD (B) patients were calculated by subtracting the test sample response achieved by stimulation with autologous LCL, LCLs matched on multiple alleles or HLA class I-restricted EBV peptide pools from the response obtained using the HLA-mismatched LCL or irrelevant peptide. The mean of the initial and peak responses were compared in a non-parametric Mann-Whitney U test. The bars and error bars represent the mean and the sd respectively.

4.2.2 Assessment of EBV-specific T cell responses in patients with EBV-positive Hodgkin's Disease

4.2.2.1 Patient Characteristics

The characteristics of the 6 patients with Hodgkin's disease (HD) are shown in **Table 4.4**. The average age at diagnosis of HD was 34.6 years and the male: female ratio was 1:1. Five patients were staged according to the Ann Arbor classification (**Appendix 1.1**) as Stage IIB or above. Three patients had an EBV-positive tumour confirmed by the presence of LMP antigen by immunohistochemistry (2=mixed cellularity, 1=nodular sclerosing histological subtypes). Within this group, 2 patients were treated with combination chemotherapy and 1 patient with localised radiotherapy. 1 patient achieved a complete clinical response to treatment, but relapsed 2 years after diagnosis and was successfully treated with an autologous stem cell transplant. The other two patients were lost to follow up and therefore clinical response to treatment is unknown.

Three patients had an EBV-negative tumour confirmed by LMP staining (1=mixed cellularity, 2=nodular sclerosing histological subtypes). All three patients were treated with combination chemotherapy. 1 patient was also treated with mediastinal radiotherapy and has remained in remission. 1 patient had disease refractory to treatment and despite further combination chemotherapy died of disease 14 months following diagnosis. The third patient was lost to further follow-up.

6 patients who were investigated for lymphadenopathy and found to have reactive/inflammatory changes on biopsy (Reactive hyperplasia) were included as histological negative controls. The average age at diagnosis was 41 yrs and the male to female ratio was 1:1.

<i>Patient Code</i>	<i>Age at diagnosis (yrs)/Sex</i>	<i>Histology</i>	<i>Stage at diagnosis</i>	<i>Site of disease</i>	<i>Primary treatment</i>	<i>Clinical response</i>	<i>Relapse treatment</i>
HD/EBV⁺ 1	29/M	MC	IIIa	Cervical and inguinal LN	ABVD	CR	Autologous Stem cell transplant (CR)
HD/EBV⁺ 2	26/F	MC	Ia	Cervical	RT	LTF	
HD/EBV⁺ 3	33/F	NS	Iib	Supraclavicular, mediastinal LN	ABVD	LTF	
HD/EBV⁻ a	39/F	MC	IIIb	Supraclavicular, mediastinal, para-aortic LN	ABVD + RT	CR	High dose chemotherapy (PD). DoD
HD/EBV⁻ b	63/M	NS	IIIb	Mediastinal, para-aortic LN	ChlVPP	SD	
HD/EBV⁻ c	19/M	NS	Iib	Supraclavicular, mediastinal LN	ABVD	LTF	

Table 4.4: Characteristics of patients who developed HD

M, male; F, female; MC, mixed cellularity histological subtype; NS, Nodular sclerosing histological subtype; LN, lymphadenopathy; RT, radiotherapy; ABVD/ChlVPP, combination chemotherapy; CR, complete clinical response; SD, stable disease; PD, progressive disease; LTF, lost to follow-up; DoD, died of disease. Patients were staged according to the Ann Arbor classification (**Appendix 1.1**). Response to treatment was measured with radiological imaging and defined according to the RECIST criteria (**Appendix 1.3**).

4.2.2.2 Comparison of EBV-specific T cell responses in EBV-positive and EBV-negative HD

The main objectives of these experiments were to determine whether the assay could detect EBV immunity in a second group of patients with malignancy and also whether any differences could be detected to explain the lack of tumour control seen in the EBV-positive HD patients.

TILs were used as this gave an opportunity to investigate the function of the EBV cellular immune response recruited to the site of the disease. Responses from TILs of HD patients were therefore compared to T cells found in patients with lymph nodes enlarged in response to inflammation (Reactive hyperplasia). For two HD patients (HD/EBV⁺ 3 and HD/EBV⁻ b), TILs were not available and therefore PBMCs were studied. These patients were analysed separately.

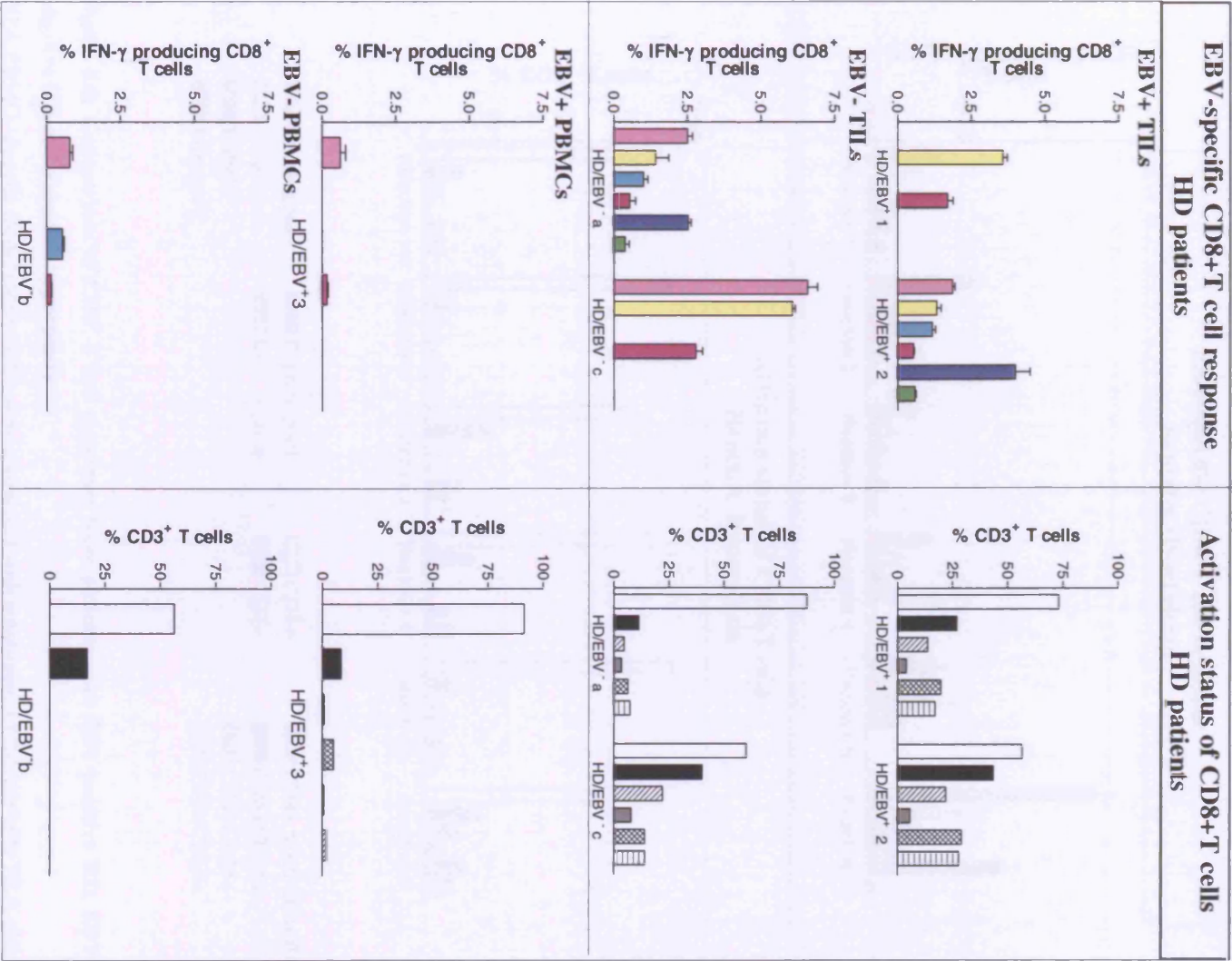
T cell responses were assessed by stimulating TILs, LN cells or PBMCs with autologous or partially HLA matched LCLs or HLA class I restricted EBV peptide pools. To focus the analysis to CD8⁺ T cell responses, LCLs that were class I matched but class II mismatched were used as stimulators. To focus the analysis to CD4⁺ T cell responses, LCLs that were matched for HLA class II alleles and mismatched at all class I alleles were used as stimulators. Peptides were not used to assess the CD4⁺ T cell response because very few class II restricted EBV epitopes have been identified. Negative controls were class I and II mismatched LCLs and an EBV peptide restricted through an irrelevant class I HLA allele plus DMSO (CD8⁺ T cell response only). The activation status of the CD4⁺ and CD8⁺ T cells was assessed by staining for MHC class II expression in all patients except HD/EBV⁻ b.

The EBV-specific CD8⁺ T cell responses together with the proportion of CD8⁺ T cells and CD8⁺MHC class II⁺ T cells from patients with EBV-positive HD, EBV-

negative HD and reactive hyperplasia are illustrated in **Figure 4.6**. Results show that a functional CD8⁺ EBV-specific response could be detected in all 12 patients tested, demonstrating that the assay could be used to assess EBV immunity in a second patient cohort.

Comparisons in the magnitude of the EBV response from TILs of EBV-positive and EBV-negative HD patients showed no significant difference using autologous LCLs, partially HLA-matched LCLs and HLA-restricted EBV peptide pools as stimulators (**Figure 4.7**). A statistically borderline difference was detected between the magnitude of the response from TILs of EBV-negative HD patients and lymphocytes from reactive LNs ($p=0.04$), however this was not observed between the magnitude of the response from TILs of EBV-positive patients compared to reactive LNs ($p=0.12$). Further examination of the T cell composition in the LNs showed that the proportion of CD8⁺ T cells in the EBV-positive HD tumours as well as the proportion expressing the activation markers CD69, CD38 and MHC class II was significantly higher than both the EBV-negative tumours and the reactive LN ($p=0.016$, $p=0.0086$, $p=0.0033$, $p=0.0116$, $p=0.0044$ for CD8, MHC class II, CD69, CD25 and CD38 respectively, **Figure 4.7**). Note activation data for patient HD/EBV^b was not available. It is known that TILs from HD patients are more activated than those from reactive hyperplasia (Amlot *et al.*, 1996). These data raise the possibility that the EBV antigens present in EBV-positive HD tumours promote recruitment of EBV-specific T cells into the lymph nodes but that this may not translate into increased anti-EBV activity as the proportion of IFN- γ producing T cells was the same as that detected in the reactive LNs and the EBV-negative TILs. Reasons for this include the presence of regulatory CD8⁺ T cells, represented by the increased proportion of CD25⁺CD8⁺ T cells in the EBV-positive TILs.

EBV-specific CD8⁺ T cell responses were much lower in the two patients where PBMCs were analyzed compared to those elicited from the four patients in which TILs were studied. This may indicate differences in the frequencies of EBV specific T cells at these two locations however definitive assessment would require analysis of both TILs and PBMCs from each patient.



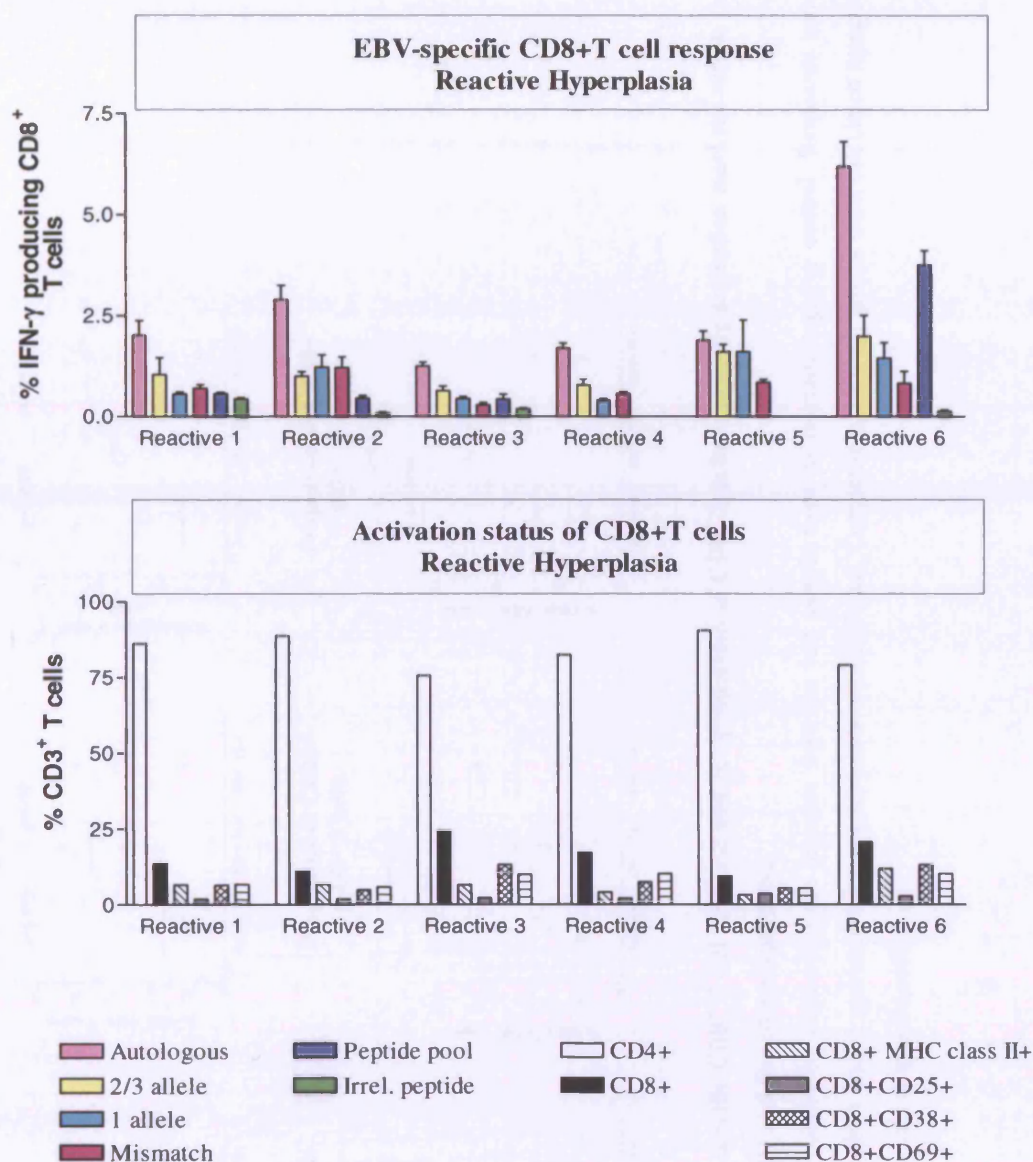


Figure 4.6: Comparison of CD8⁺ T cell responses from patients with EBV-positive HD, EBV-negative HD and Reactive hyperplasia

TILs, PBMCs or cells from reactive LNs were stimulated with autologous LCLs, partially HLA class I matched LCLs or HLA class I restricted EBV peptides. EBV-specific T cells were quantified by measuring IFN- γ production by CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells. The number of CD8⁺ T cells present in each sample was expressed as a percentage of the CD3⁺ T cells. CD4⁺ and CD8⁺ T cells and CD8⁺ T cells expressing MHC class II, CD25, CD38 or CD69 were represented as a percentage of the CD3⁺ T cells. The bar and error bar values represent the mean and sd values respectively.

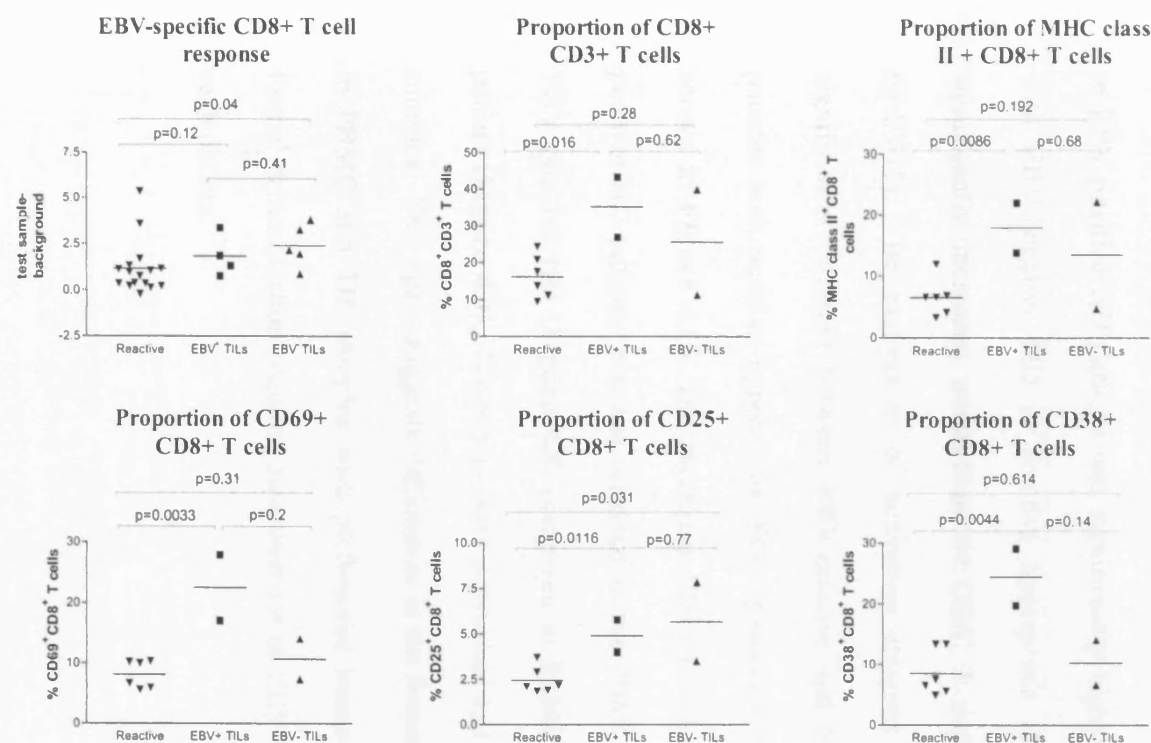
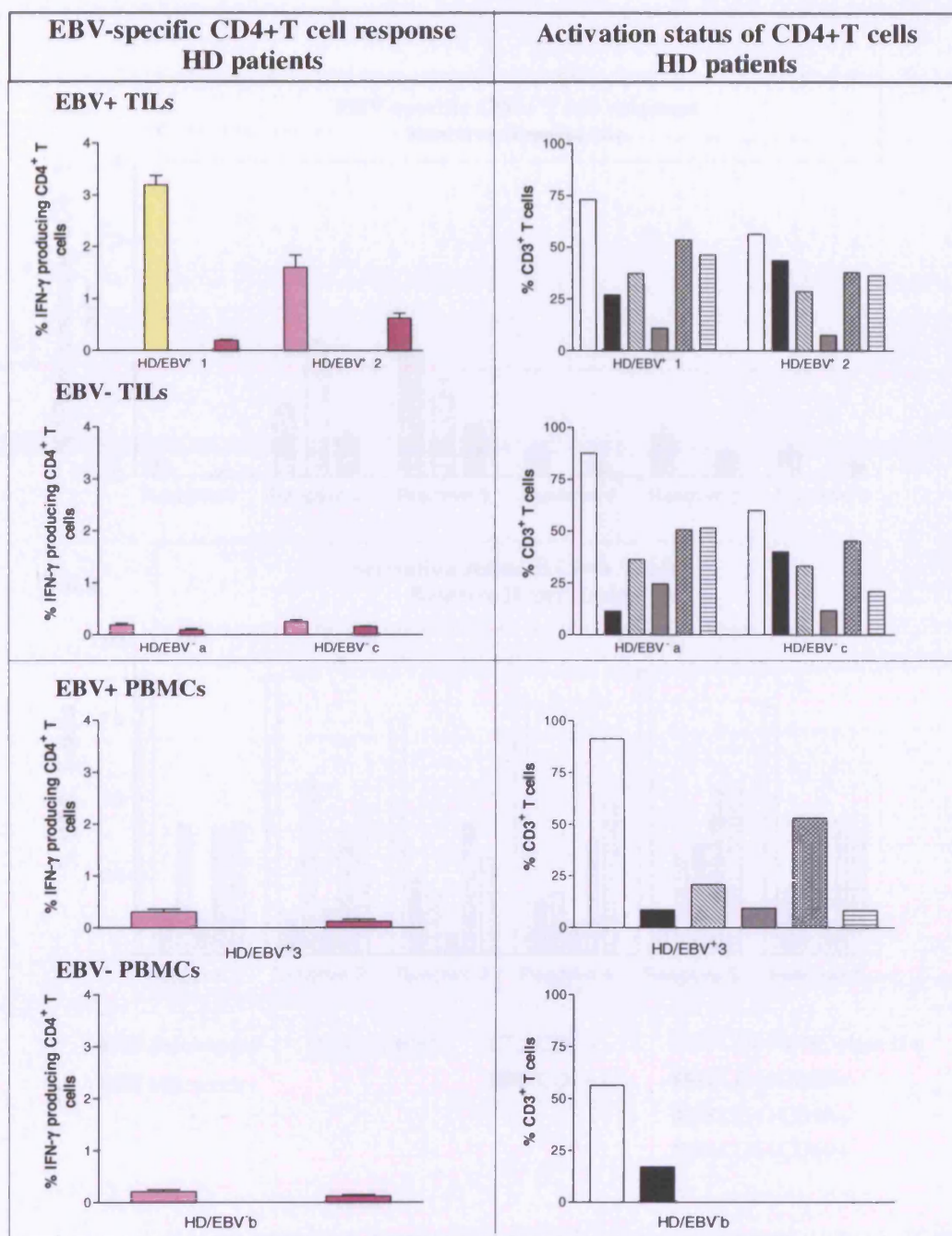


Figure 4.7: Comparison of the magnitude of the EBV-specific CD8⁺ T cell response and the proportion of CD8⁺ T cells expressing activation markers within the LNs of patients with EBV-positive and EBV-negative HD and Reactive Hyperplasia

Magnitude of the EBV-specific T cell response was calculated by subtracting the response from the test sample from its relevant negative control. Responses using autologous LCLs, partially HLA class I matched LCLs or HLA class I restricted EBV peptide pools were included. Comparisons between groups were calculated using an un-paired t-test and expressed as a p value. Note different y axis ranges are used.

The CD4⁺ T cell responses (assessed by measuring IFN- γ production from CD3⁺CD8⁻ T cells) from patients with EBV-positive HD, EBV-negative HD and reactive hyperplasia are illustrated in **Figures 4.7 and 4.8**.

Results show the assay was able to detect CD4⁺ T cell responses to EBV in addition to CD8⁺ EBV specific T cells. The magnitude of the EBV response from CD4⁺ TILs in EBV-positive HD patients was significantly higher than the response in patients with EBV-negative HD or reactive hyperplasia (**Figure 4.9** $p=0.014$), despite significantly increased proportions of CD4⁺ T cells in the reactive LN group ($p=0.016$). The expression of activation antigens on CD4⁺ T cells was not significantly different between EBV-positive and EBV-negative HD patients or patients with reactive hyperplasia. Flow cytometry data representing this result is shown in **Figure 4.10**. The increased CD4⁺ T cell response in TILs from EBV-positive HD patients was not reflected in the PBMC samples from a patient with EBV positive HD (**Figure 4.8**) compared to PBMCs from an EBV-negative HD patient (**Figure 4.8**). Responses were equivalent and relatively low in both PBMC samples. This again suggests differences in the frequencies of EBV specific T cells in PBMC and TIL samples with preferential homing to EBV-positive TILs, but formal demonstration requires measurement of EBV immunity at both locations in each patient.



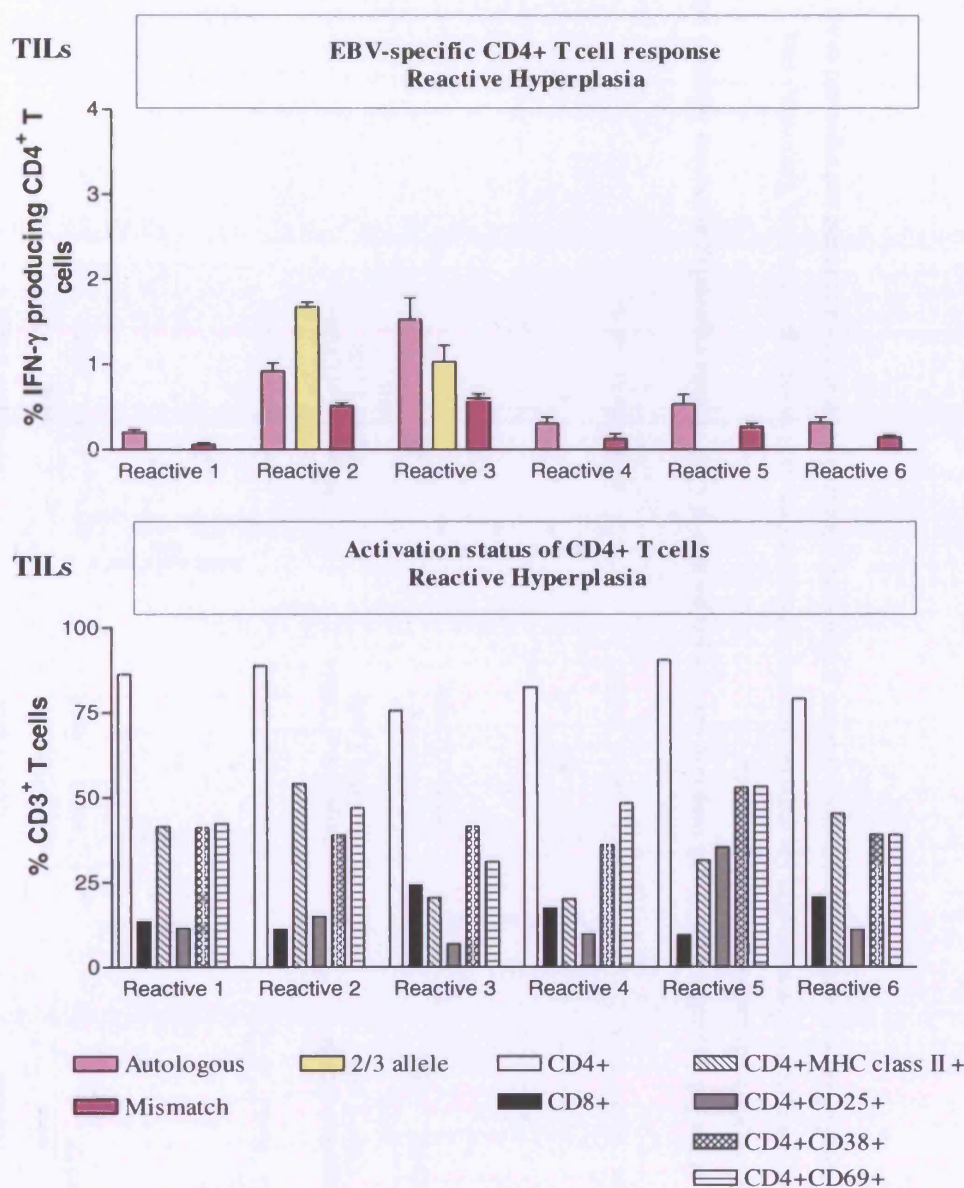


Figure 4.8: Comparison of CD4⁺ T cell responses in patients with EBV-positive HD, EBV-negative HD and Reactive hyperplasia

TILs, LNs or PBMCs were stimulated with autologous LCLs, or partially HLA-class II matched LCLs. EBV-specific CD4⁺ T cells were quantified by measuring IFN- γ production by CD8⁺ T cells using intracellular cytokine staining, and expressed as percentages of CD3⁺CD8⁺ T cells. CD4⁺ and CD8⁺ T cells and CD4⁺ T cells expressing MHC class II, CD25, CD38 or CD69 were represented as a percentage of the CD3⁺ T cells. The bar and error bar values represent the mean and sd values respectively.

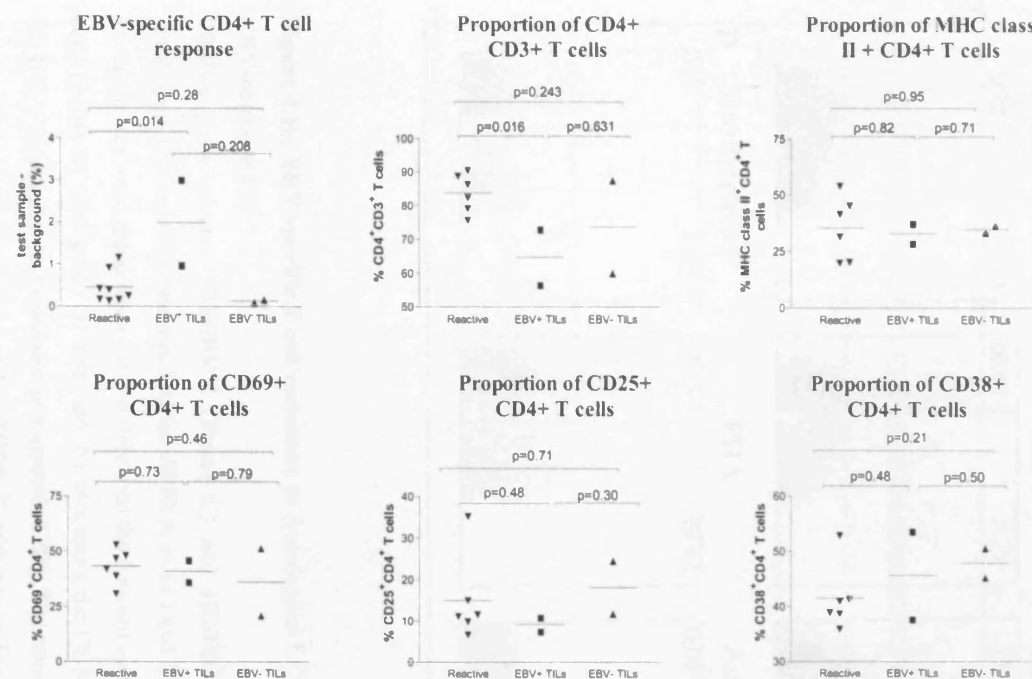


Figure 4.9: Comparison of the magnitude of the EBV-specific CD4⁺ T cell response and the proportion of CD4⁺ T cells expressing activation markers within the LNs of patients with EBV-positive and EBV-negative HD and Reactive Hyperplasia

Magnitude of the EBV-specific T cell response was calculated by subtracting the response from the test sample from its relevant negative control. Responses using autologous LCLs or partially HLA class II matched LCLs were included. Comparisons between groups were calculated using an un-paired t-test and expressed as a p value. Note different y axis ranges are used.

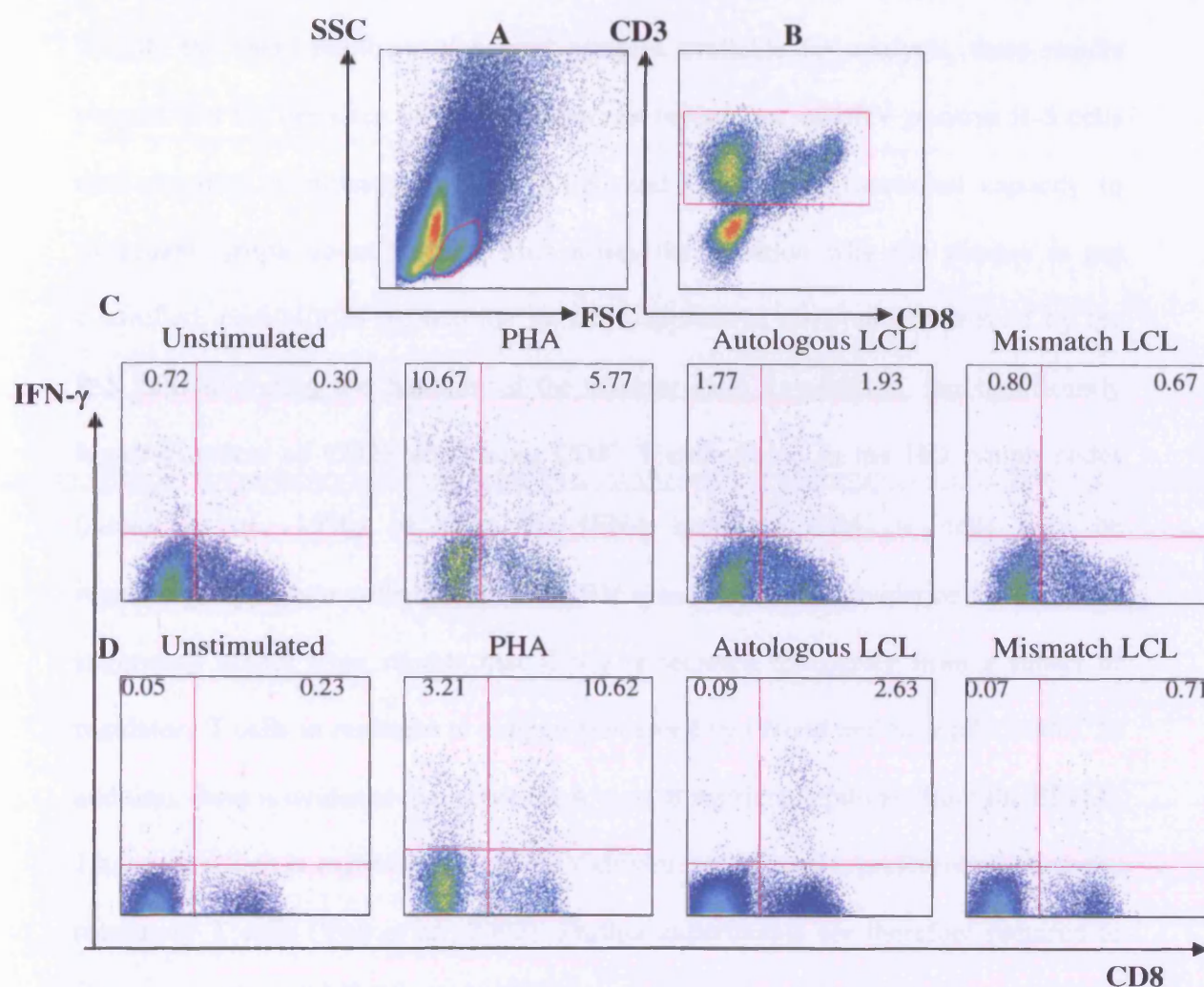


Figure 4.10: EBV-specific T cell responses to Autologous LCLs in patients with EBV-positive and EBV-negative HD

PBMCs from patients HD/EBV⁺2 (**Panel C**) and HD/EBV⁻a (**Panel D**) were stimulated with autologous LCLs. LCLs mis-matched for all HLA class I and II alleles were used as negative controls. Lymphocytes were gated from the cell debris on the forward scatter/side scatter plot (**panel A**). CD3⁺ T cells (**panel B**) were gated on a second dot plot using the CD3/CD8 plot. T cells were then assessed for IFN- γ production in a third dot plot (**panels C/D**). The numbers in the upper left quadrant of each panel represent the percentage of CD3⁺CD8⁻ T cells producing IFN- γ and in the upper right quadrant the percentage of CD3⁺CD8⁺ T cells producing IFN- γ .

Despite the small numbers of patient samples available for analysis, these results suggest that the presence of EBV antigens on the surface of EBV positive R-S cells does promote recruitment of both CD8⁺ and CD4⁺ with functional capacity to malignant lymph nodes in HD. This raises the question why the disease is not controlled; possibilities include the immunosuppressive environment created by the R-S cells inhibiting the function of the effector cells. In addition, the significantly higher numbers of CD25 expressing CD8⁺ T cells found in the HD lymph nodes (Amlot *et al.*, 1996) or even the IFN- γ secreting CD4⁺ T cells may be regulatory/suppressor cells that inhibit EBV specific T cells. Evidence for the latter suggestion comes from reports that IFN- γ is secreted transiently from a subset of regulatory T cells in response to antigen (reviewed by (Wood and Sawitzki, 2006)). In addition, there is evidence that some HLA class II restricted epitopes from the EBNA-1 antigen, which is expressed by all EBV-driven malignancies, preferentially activate regulatory T cells (Voo *et al.*, 2002). Further experiments are therefore required to characterise the CD25⁺ T cells and IFN- γ producing CD4⁺ TILs stimulated by EBV antigens in this assay.

Comparison of the different EBV antigens used in the assay for patients with HD and reactive hyperplasia showed that autologous LCLs were able to stimulate a positive EBV-specific CD8⁺ T cell response in all 11 patients tested. 2/3 partially HLA allele matched LCLs were also able to stimulate a positive response in all patients with HD, and 5 out of 6 patients with reactive hyperplasia. Screening with LCLs matched on single alleles however produced variable results, stimulating a positive response in only 5 out of 9 patients tested despite all responding to autologous LCLs. HLA-restricted EBV peptide pools were only tested in 6 patients due to limited cell

numbers but in all cases stimulated a positive CD8⁺ T cell response. These results are consistent with those obtained from healthy individuals and PTLN patients and confirm that autologous LCLs are the most reliable source of EBV antigen. In the absence of these cells, LCLs matched on at least 2 alleles or HLA-restricted peptide pools tailored to patient HLA type represent alternative sources of antigens.

Autologous LCLs and LCLs matched on at least 2 class II alleles were also able to stimulate CD4⁺ T cell responses in patients with EBV-positive and EBV-negative HD as well as reactive hyperplasia showing that this assay is an effective approach for measuring CD4⁺ as well as CD8⁺ EBV specific T cell immunity.

4.3 DISCUSSION

Results from work in this chapter established that the clinical assay developed in Chapter 3 to measure IFN- γ producing EBV-specific T cells in healthy individuals could be applied to measure EBV immunity in EBV related disease states.

In patients with PTLT, the main objective was to determine whether the assay could be used to measure any increase in the functional cellular immune response to EBV during treatment with RIS. Monitoring changes in cell surface expression of activation markers such as MHC class II on CD8⁺ T cells has been used successfully for this purpose in PTLT patients (Amlot *et al.*, 1996; Rees *et al.*, 1998; Amlot *et al.*, 2007; Baudouin *et al.*, 2004), however the main disadvantage of this approach is that it is unable to provide information regarding the antigen specificity of the cellular immune response. Work presented in Chapter 3 showed that functional EBV-specific T cells could be measured in healthy individuals after a short *in vitro* stimulation with autologous LCLs or HLA-restricted peptide pools. Developing this assay for use in the context of PTLT would therefore address the limitations of the cell phenotyping assay.

Initial experiments looked at 12 PTLT patients with EBV-positive and EBV-negative tumours and examined the EBV-specific T cell response from serial PBMC samples stimulated *in vitro* with autologous LCLs, partially HLA matched LCLs or EBV HLA-restricted peptide pools.

Results from EBV-positive PTLT patients showed that the assay was able to detect emergence of IFN- γ producing EBV-specific CD8⁺ T cells in response to reduction in immunosuppression and that this was associated with clinical response in all 8

patients tested. This finding confirms that these cells play a critical role in the elimination of malignant B cells transformed by EBV. The importance of IFN- γ producing T cells in EBV immunity is beginning to be appreciated with the finding that previously undetected EBNA-1 specific CD8⁺ T cells (Levitskaya *et al.*, 1995) do exist and produce IFN- γ rather than lytic activity (Lee *et al.*, 2004). IFN- γ production may therefore be a more reliable indicator of target cell recognition by EBV-specific T cells rather than lytic activity determined by conventional cytotoxicity assays.

EBV-specific T cell responses also mirrored the changes in MHC class II expression on the surface of the effector cells but typically lagged behind the initial increase in activation marker expression and declined more rapidly. A similarly short duration for expansion of an EBV specific T cell response has previously been seen in IM (Callan *et al.*, 1996). The marked difference in duration indicates that the increase in MHC Class II expression on activated CD8⁺ T cells not only encompasses the EBV specific T cell response but must also include a wider spectrum of T cell activation as immunosuppression is reduced that may include regulatory T cells. A rise in MHC class II expression is currently used to guide step-wise reduction of immunosuppression in patients with PTLN (Amlot *et al.*, 2007), however replacement of this non-specific measurement by an EBV specific methodology is likely to increase the accuracy. The advantage of an assay which measures IFN- γ producing T cells in response to EBV antigen stimulation is that it provides a specific measure of EBV immunity. Further studies using a larger cohort of PTLN patients may also determine a threshold number of IFN- γ producing EBV-specific CD8⁺ T cells in the peripheral blood that are required to control PTLN.

Although the majority of EBV-positive PTLT patients respond to treatment RIS, a small percent will be unresponsive or relapse early. Reasons for this are not known but suggest that PTLT tumours are capable of evading the EBV-specific T cell response perhaps by down-regulating some or all of the surface antigens. A possible example of this is seen in patient EBV⁺ 3 who initially achieved a partial response during RIS but relapsed soon after and was then treated with chemotherapy. Immunosuppression in this patient was withdrawn over a protracted time scale and not according to standard protocol (Amlot *et al.*, 2007). This may have inadvertently provided a window in which the tumour had time to adapt by either down-regulating the EBV antigens targeted by the slowly emerging EBV immune response or outgrowth of cells within the tumour which had a restricted pattern of EBV antigen expression. The fall in the EBV-specific immune response observed in this patient on relapse could either represent the decline of the effector CD8⁺ T cells specific for the immunodominant antigens, or result from immunosuppression induced by cytotoxic chemotherapy.

Evidence that PTLT tumours are able to down regulate EBV antigens from their surface is supported by studies that have shown development of PTLT lesions from EBV transformed atypical post-germinal B cells (Timms *et al.*, 2003). This finding is analogous to the pathogenesis of EBV-positive HD, a tumour that has evolved secondary to genetic mutations and down-regulated EBV antigen expression to LMP-1, LMP-2 and EBNA-1 (Re *et al.*, 2005). Such a connection between EBV-positive PTLT and HD therefore suggests that EBV-positive PTLT can also employ similar immune evasion strategies and down regulate immunodominant EBV antigens from its surface. Indeed, the common misunderstanding that PTLT expression of EBV

antigens is comparable to LCL and latency type III has been refuted by studies showing a position somewhere between latency type II and III with loss of some of the more immunogenic EBV antigens (Timms *et al.*, 2003; Cen *et al.*, 1993). Both of the aforementioned studies found variable loss of EBNA 2, 3 or LMP1. Currently, PTLD lesions are simply classified on EBV status according to the presence of EBER or EBNA-1 on immunohistochemical staining. It is known that staining for LMP1/2 is highly unreliable for determining EBV status and if present is usually only on a minority of PTLD cells. False-negative results are therefore possible. Quantifying EBV gene expression by molecular techniques such as qRT-PCR on individual tumours as well as identifying those PTLD tumours that are derived from atypical post-germinal B cells and are more likely to change EBV expression, could therefore provide valuable prognostic information.

EBV-negative PTLD tumours as well as EBV-positive Hodgkin's like PTLD typically develop much later than EBV-positive tumours of large B cell type. These tumours may represent a continuation of the immune evasion strategy described above, but with a longer lead time enabling complete down regulation of all EBV antigens. It was anticipated therefore that restoring the EBV-specific cellular immune response by withdrawing immunosuppression in these patients would have no effect on controlling tumour proliferation or clinical response. The results from all four patients with EBV-negative PTLD confirmed this proposal, demonstrating that although EBV-specific T cells could be detected in EBV-negative PTLD patients, their frequency remained unchanged after RIS. Likewise CD8⁺MHC class II⁺ expression was also unaltered.

Treatment with RIS can be effective however, in a small minority of EBV-negative PTLD patients and is often associated with a transient rise in the activation marker

CD69 (P. Amlot, personal communication). Expression of other activation T cell antigens such as CD25 and MHC class II remains unchanged. This suggests that RIS may allow restoration of an anti-tumour T cell response leading to tumour control.

The main objective in patients with HD was to demonstrate whether the assay could be used to evaluate EBV immunity in patients with HD and whether this could provide any explanation for ineffective tumour control in EBV-positive disease.

Analysis of CD8⁺ T cells from EBV-positive and EBV-negative tumours showed that the proportion of CD8⁺ T cells expressing activation antigens was significantly increased in the EBV-positive tumours. In addition, EBV-specific CD8⁺ T cell responses were also detected but there was no difference in the magnitude of the responses detected from EBV-positive or reactive lymph nodes. This suggests that functional EBV-specific CD8⁺ T cells are recruited to the EBV-positive tumours. Possible reasons for why this response is ineffective in controlling the disease may be the immunosuppressive environment created by the R-S cells or the presence of regulatory T cells within the LN. Another explanation may be that the TILs do not contain or have insufficient numbers of functional T cells specific for the limited set of EBV antigens expressed by the malignant R-S cells. The *in vitro* assay for EBV immunity uses stimulator cells that express the complete set of EBV antigens. The response measured is therefore likely to comprise a polyclonal memory response directed to the immunodominant EBNA 3A-C antigens (Hislop *et al.*, 2001; Rickinson and Moss, 1997) with a smaller response to the LMP1, 2 and EBNA-1 EBV antigens expressed by R-S cells in HD. If the functional EBV reactive T cells detected in TILs are specific for EBNA 3A-C, they will not be able to recognise and eliminate R-S cells. However, a problem with this proposal is how to explain

recruitment of EBNA 3A-C specific T cells to malignant LNs if the antigens are not expressed. HLA-restricted peptide pools containing class I and II epitopes from LMP1, LMP2 and EBNA1 antigens would be helpful in determining the precise EBV antigen specificity of TILs but relatively few of these peptides have been described.

The results also showed an accumulation of CD4⁺ T cells in tumours expressing EBV antigens and presence of EBV specific CD4⁺ T cells among these TILs. This may represent an increased EBV specific T_H1 response or an increased antigen-induced T regulatory response or a combination of both. Further experiments to address the balance between T_H1 CD4⁺ T cells and antigen-induced regulatory cells include analysing the full spectrum of cytokines produced from the EBV-specific CD4⁺ T cells, as well as other markers associated with antigen-induced regulatory T cells such as FoxP3. Definitive evidence for an increased antigen-induced regulatory T cell response in EBV-positive tumours however, would be from suppression of CD8⁺ EBV-specific T cell response in functional proliferation assays.

Comparisons between the EBV-specific T cell responses generated from stimulation with different EBV antigens in the context of PTLN and HD revealed that autologous LCLs were the most reliable at detecting a positive response. Autologous LCLs however, could not always be successfully generated despite repeated attempts. In this situation, a positive EBV-specific response was detected in the majority of patients by stimulation with LCLs matched for at least 2 HLA alleles or using HLA class I-restricted EBV peptides pools. Importantly, these results were achieved using a cohort of PTLN patients that expressed a diverse spectrum of HLA alleles (6 different HLA-A, 11 different HLA-B and 11 different HLA-Cw alleles).

In order for this assay to be successful clinically as a method of monitoring emergence of EBV immunity in PTLD patients, autologous LCLs should either be generated pre-emptively in all patients undergoing solid organ transplantation anticipating they will be needed if PTLD occurs or PBMC should be stored frozen prior to transplantation so that EBV transformation of B cells can be attempted if PTLD occurs. These approaches make use of PBMCs collected before transplant so the cells are not altered by immunosuppressive drugs thus maximising the chance of successful transformation. These pre-emptive measures also ensure that an autologous LCL is readily available immediately on, or shortly after, diagnosis of PTLD. In patients where autologous LCLs cannot be generated, possible options include testing PBMCs with several LCLs matched on multiple HLA alleles and identifying the one capable of eliciting the maximal EBV-specific T cell response. This LCL could then be cryopreserved and readily retrieved in the event of a PTLD diagnosis. Alternatively, HLA class I-restricted peptide pools could be used, but due to limited knowledge of the epitopes presented by less common HLA alleles and alleles present in non-Caucasoid populations, this approach could not be used for all individuals.

In conclusion, work described in this chapter demonstrates that EBV-specific T cell responses can be rapidly and reliably measured in patient samples. In the context of PTLD, EBV-specific T cell responses were shown to increase as immunosuppression was withdrawn and correlate with current methods of measuring recovery of EBV immunity, thereby demonstrating the clinical utility of the assay. Recovery of EBV immunity was also shown to correlate with control of the PTLD providing evidence that IFN- γ producing CD8⁺ T cells are directly involved in mediating effective tumour control.

Experiments with EBV-positive HD showed no evidence for absence of EBV-specific immunity in the affected LNs, thus failure of the immune system to control disease is not due to absence of protective T cells. This finding supports accumulating evidence that localized suppression of the immune system may occur mediated either by factors produced by the malignant R-S cells or presence of regulatory T cells that suppress the anti tumour response. Limited patient samples prevented a more detailed examination of these possibilities.

Chapter 5

**Assessment of quantitative real-time polymerase
chain reaction (qRT-PCR) to measure IFN- γ
production by EBV-specific T cells using small cell
numbers.**

5.1 INTRODUCTION

Experiments presented in Chapters 3 and 4 established that detection of functional EBV-specific T cells could be achieved by stimulation with autologous LCLs in both healthy individuals and patients with EBV-driven malignancies using a flow-cytometry based ICS assay. This method was chosen for a clinical assay due to its superiority over other methods in terms of reproducibility, greater signal-to-noise ratio, speed of generating results, cost effectiveness and ease of standardisation.

The main limitation with ICS is the relatively large number of PBMCs required to detect a positive EBV-specific T cell response. Detection of a positive response requires 4 samples (autologous LCL or multiple HLA-allele matched LCL (test)), HLA-mismatched LCL (negative control), PHA stimulated PBMCs (positive control) and unstimulated PBMCs (negative control) and thus a total of 4×10^6 PBMCs. However, PBMCs obtained from patients with PTLD are often limited in numbers and viability (**Table 4.2:** mean number and [range] = 9.22×10^6 [$2.2 \times 10^6 - 20.5 \times 10^6$] and viability = 94% [87-98%]). Therefore, a method that could reliably detect EBV-specific T cell responses using fewer PBMCs would be desirable.

Another limitation with ICS is that the number of parameters that can be examined for each sample is restricted by the number of fluorescent detectors available on the flow cytometer. Although the latest flow cytometers are capable of detecting up to 18 different fluorochromes (<http://www.bdbiosciences.com/index.shtml>), most flow cytometers currently used in clinical immunology laboratories are limited to detection of three fluorochromes. In the context of the flow cytometry based assay for detection of EBV immunity, this equates to CD8 FITC, IFN- γ PE and CD3 Cy5.5.

Therefore only one cytokine can be studied per sample. Although IFN- γ production by CD8⁺ T cells was found to be the most effective cytokine at detecting an EBV-specific T cell response in healthy individuals (**Figure 3.2**), a method which is able to detect several cytokines from one sample would have the advantage of providing additional information regarding T cell functionality. Furthermore it would be advantageous to examine patterns of cytokine production in EBV-driven malignancies such as HD and NPC where evidence indicates EBV exploits immune evasion strategies based on inducing aberrant cytokine profiles (Marshall *et al.*, 2004; Skinnider and Mak, 2002; Kis *et al.*, 2006; Hu *et al.*, 2004; Budiani *et al.*, 2002).

Quantitative real-time PCR (qRT-PCR) is a highly sensitive molecular method for measuring the levels of mRNA transcripts of genes of interest. This technique detects gene expression from a very small number of cells and multiple parameters can be studied for each sample. Therefore use of this technique may circumvent the limitations of ICS, however expensive instrumentation that is not routinely available in clinical immunology laboratories is required which may limit utility.

The principle of qRT-PCR is that following the initial conversion of RNA into cDNA template by an RNA-dependent DNA polymerase (reverse transcriptase), specific segments of DNA encoding target genes of interest are amplified by PCR and these products are then detected and quantified as they accumulate (i.e. in 'real-time') using fluorescent reporter molecules. This technology therefore enables reliable measurement of target gene expression from small cell numbers. Two types of detection chemistries are used in qRT-PCR. The non-probe based method quantitates the production of amplicon using a non-sequence specific fluorescent DNA dye such as SYBR green I. This dye exhibits little fluorescence in solution but emits a strong

fluorescent signal upon binding to double-stranded DNA (Morrison *et al.*, 1998). Fluorescence therefore increases as amplification proceeds. Disadvantages of this detection method however, are two-fold. Quantitation requires extensive optimisation using a melting point curve or dissociation analysis (Ririe *et al.*, 1997) and secondly preferential binding of SYBR Green to specific DNA sequences during amplification interferes with PCR kinetics rendering multiplexing (simultaneous analysis of multiple target genes from the same sample) problematic. Probe-based chemistries such as TaqMan[™] probes use amplicon-specific fluorescent probes that only generate a fluorescent signal if the probe hybridises with its complementary DNA sequence (**Figure 2.4**). This technique therefore has an advantage over the non-probe based method by introducing an additional level of specificity. Furthermore, multiplex qRT-PCR is possible using probes labelled with different fluorophores (Grace *et al.*, 2003). Given these advantages, a probe-based chemistry was chosen for the development of a qRT-PCR assay to measure EBV-specific immunity.

Another important consideration in the design of a qRT-PCR assay is the quantitation strategy. Absolute quantitation determines the copy numbers of the transcript of interest by relating the PCR signal from the test sample to a standard curve. While this method is useful for quantifying viral or tumour load in patient samples, it has the inherent disadvantage of requiring production and accurate quantitation of cDNA to generate a standard curve which should be included in every experiment (Pfaffl and Hageleit, 2001). Relative quantitation however compares the changes in expression of a target gene relative to an internal reference gene. The advantage of relative quantitation over absolute quantitation is that it eliminates the need for standard curves to be included into each experiment thus increasing throughput. Relative

quantitation was chosen for the assessment of IFN- γ mRNA expression by EBV-specific T cells because an absolute measurement was not required for interpretation of results. The important parameter was the difference in IFN- γ expression between the test sample (EBV stimulated PBMCs) and the appropriate negative control (unstimulated PBMCs or irrelevant antigen stimulated PBMCs). Calculations were performed using the comparative Ct method ($\Delta\Delta$ Ct (Livak and Schmittgen, 2001)) and is detailed in **Chapter 2, section 2.4.5**.

Current clinical applications of qRT-PCR exploit its ability to detect gene expression from small amounts of material and is the method used to detect minimal residual disease (MRD) in a variety of haematological malignancies (Marcucci *et al.*, 1998; Elmaagacli *et al.*, 2000; Hosler *et al.*, 1999; Ladetto *et al.*, 2001) and EBV viral load (Hochberg and Thorley-Lawson, 2005a; Stevens *et al.*, 2005; Ryan *et al.*, 2004). Therefore the technique of qRT-PCR is increasingly being implemented in clinical diagnostic laboratories.

Recent research has also illustrated the potential use of qRT-PCR in monitoring T cell mediated immune responses to tumour antigens. Kammula *et al* used qRT-PCR to detect increased melanoma-specific T cell responses in the peripheral blood of melanoma patients following vaccination with melanoma HLA class I-restricted peptides (Kammula *et al.*, 1999; Kammula *et al.*, 2000). PBMCs from vaccinated individuals were briefly stimulated *in vitro* with the peptides present in the vaccine and cytokine expression (IFN- γ , TNF- α , IL-2, GM-CSF) or activation antigen expression (CD69 and CD25) assessed. Similar studies have also been used to detect low frequencies of leukaemia-specific CD8⁺ T cells in patients with CML following HSCT (Rezvani *et al.*, 2003) as well as endogenous tumour associated antigen

(TAA)-specific T cells in breast cancer patients at the time of diagnosis (Rentzsch *et al.*, 2003). Research studies have also taken advantage of the ability to detect gene expression by qRT-PCR from small amounts of material to investigate immune interactions within the tumour microenvironment after vaccination (Ohnmacht *et al.*, 2001). Such studies are difficult with immunohistochemistry or flow cytometry due to the relatively large amounts of material required. Analysis of the immune response at the site of pathology has the advantage of examining the effector populations within the tumour which may be different from the circulating lymphocytes in PBMCs. Analysing cytokine expression within the tumour microenvironment by qRT-PCR has been used successfully to investigate the pathogenesis of both Hodgkin's lymphoma (Malec *et al.*, 2004) and melanoma (Kammula *et al.*, 1999; Mocellin *et al.*, 2001).

An important factor to consider with qRT-PCR assays is that it measures mRNA transcript expression and not protein. Therefore a concern with using qRT-PCR to measure cellular response to antigen is whether cytokine mRNA transcription correlates with the production of cytokine protein. To address this concern, positive correlations have been made with the mRNA transcript levels of IL-2, IL-4, TGF- β_1 and IL-10, and concentrations of the corresponding proteins detected by ELISA in PBMCs stimulated with anti-CD3 mAb (OKT3) (Mocellin *et al.*, 2003), melanoma peptides (Kammula *et al.*, 2000) and the purified protein derivative of tuberculin (PPD, (Ekerfelt *et al.*, 2002)). In experimental situations, Rezvani *et al* measured the CD8⁺ T cell response following stimulation with a CMV peptide and demonstrated a good correlation between mRNA IFN- γ transcripts measured by qRT-PCR and the number of CMV-specific CD8⁺ T cells detected by tetramer analysis (Rezvani *et al.*,

2003). These studies show that cytokine mRNA expression can be used as an appropriate indicator of functional cytokine producing cells.

The ability of qRT-PCR to reliably detect functional T cells has also been compared with that of other assays. qRT-PCR has been shown to be capable of detecting very low frequencies of antigen specific T cells (range 1 in 50,000 to 1 in 100,000, (Kammula *et al.*, 1999; Rezvani *et al.*, 2003) compared to a detection limit of 1 in 10,000 cells for tetramer analysis (Rezvani *et al.*, 2003; Klenerman *et al.*, 2002). A comparison of ELISA, ELIspot, ICS and qRT-PCR to measure production of IFN- γ by CD8⁺ T cells specific for tetanus toxoid antigen in healthy individuals vaccinated 5 years previously has been performed (Tassignon *et al.*, 2005). The frequency of tetanus-specific T cells was low with each method used, however the probability of detecting a true positive response (sensitivity) by qRT-PCR was 71% compared to 50% by ICS. The highest sensitivity was seen with ELIspot (93%). One explanation for the differences in sensitivity between ELIspot and qRT-PCR was the 20 hour period of stimulation used in this study prior to measuring the antigen-specific response. IFN- γ mRNA transcription has been shown to increase rapidly after stimulation, peaking between 4 and 8 hours (Abdalla *et al.*, 2003; Stordeur *et al.*, 2002). Transcription levels then decline due to translation into protein. Therefore although at 20 hours accumulation of IFN- γ producing T cells could be detected by ELIspot, mRNA transcript levels may have begun to decline leading to under-estimation of IFN- γ production by qRT-PCR.

No studies to date have used qRT-PCR to measure EBV-specific T cell immunity. The aims of the work described in this chapter were to develop an assay using qRT-PCR that could be used to quantify the cellular immune response to EBV in healthy

individuals and patients with EBV-driven malignancies. Important points that were addressed were whether qRT-PCR could reliably measure IFN- γ producing T cells using smaller numbers of cells than required for ICS and whether the IFN- γ mRNA expression correlated with the frequency of IFN- γ producing EBV-specific T cells measured by ICS. In addition, the sensitivity of qRT-PCR compared to ICS was investigated by assessing whether qRT-PCR could improve differentiation of a positive signal from background in situations where a borderline response was obtained by ICS.

The assay was initially optimised with PBMCs from healthy individuals that had been stimulated with EBV HLA-restricted peptide pools. The number of mRNA IFN- γ transcripts after stimulation with EBV peptide pools or EBV-transformed LCLs were then directly compared to the frequency of IFN- γ producing CD8⁺ T cells detected by ICS in both healthy individuals and PTLD patients undergoing RIS therapy.

5.2 RESULTS

5.2.1 Assessment of a qRT-PCR assay to detect and quantify IFN- γ production by EBV-specific T cells from limited cell numbers

Initial experiments were performed to ascertain whether fewer numbers of cells could be used to detect a positive EBV-specific T cell response by qRT-PCR than required for ICS analysis. The ability to discriminate between a positive response and a negative response by qRT-PCR and ICS was also assessed using combinations of HLA class I-restricted EBV peptide pools.

5.2.1.1 *Determination of optimal duration of antigen stimulation required for the detection of IFN- γ mRNA expression by EBV-specific T cells*

Previous studies have shown that the kinetics for cytokine mRNA expression differs with the cytokine measured and the antigen used to stimulate a response. Published studies using polyclonal activation of PBMCs with PHA have reported that IL-2 and IFN- γ mRNA expression peaked 4 hours after stimulation and then rapidly fell to low levels by 16 hours (Kruse *et al.*, 2001; Stordeur *et al.*, 2002). Early induction of IFN- γ mRNA expression in CD8⁺ T cells was also seen after stimulation with tetanus toxoid and influenza antigens in healthy individuals (Kruse *et al.*, 2001). The kinetics of IFN- γ mRNA expression in response to mycobacterial antigens, however, was slower to develop. IFN- γ copy numbers peaked at 16-24 hours of stimulation in patients with active *M. tuberculosis* and were still detected at 96 hours (Listvanova *et al.*, 2003).

In order to characterise the kinetics of IFN- γ mRNA production in response to stimulation with EBV antigens, PBMCs from an EBV sero-positive HLA-A*0201

individual were stimulated with a pool of HLA-A*0201 restricted EBV peptides and compared to stimulation with PHA (positive control) or an EBV peptide restricted through an irrelevant HLA class I allele (negative control). The results are shown in **Figure 5.1** and represent the IFN- γ mRNA expression relative to unstimulated PBMCs after normalisation to the internal reference gene, GAPDH.

In order for relative quantitation to be effective it is vital that the internal reference gene is expressed at a constant level in all test samples (Karge, III *et al.*, 1998). β -actin, GAPDH, 18S ribosomal RNA and hypoxanthine-guanine phosphoribosyl transferase (HPRT) are typically used. However their expression has been shown to vary between individuals (Piechaczyk *et al.*, 1984; Stout *et al.*, 1985) and between different cell types within the same individual (Blomberg *et al.*, 1987; Bustin, 2000). Experiments are therefore required to validate the choice of reference gene, by screening several different candidate genes and analysing variability between samples (Dheda *et al.*, 2004).

Previous experiments performed in the laboratory (Dr K Matthews, unpublished data) compared the expression of 18S ribosomal RNA, GAPDH, HPRT and CD8 in CD3⁺CD8⁺ T cells that had been stimulated with PHA. Unstimulated CD3⁺CD8⁺ T cells were used as a negative control. Results were interpreted using the BestKeeper[®] Software (<http://www.gene-quantification.de/BestKeeper-1.zip>, (Pfaffl *et al.*, 2004)), a statistical program which selects the least variable gene by comparing the Ct values and the geometric mean of the expression of several reference genes. This showed that GAPDH mRNA expression was the least variable gene for these *in vitro* assay conditions and was therefore chosen as the reference gene for development of this assay.

Maximal induction of IFN- γ mRNA expression occurred 6-8 hours after stimulation with PHA (**Panel A**) and 4 hours after EBV-specific stimulation with a HLA-A*0201 peptide pool (**Panel B**). Therefore a 4 hour antigen stimulation period was used for all subsequent experiments.

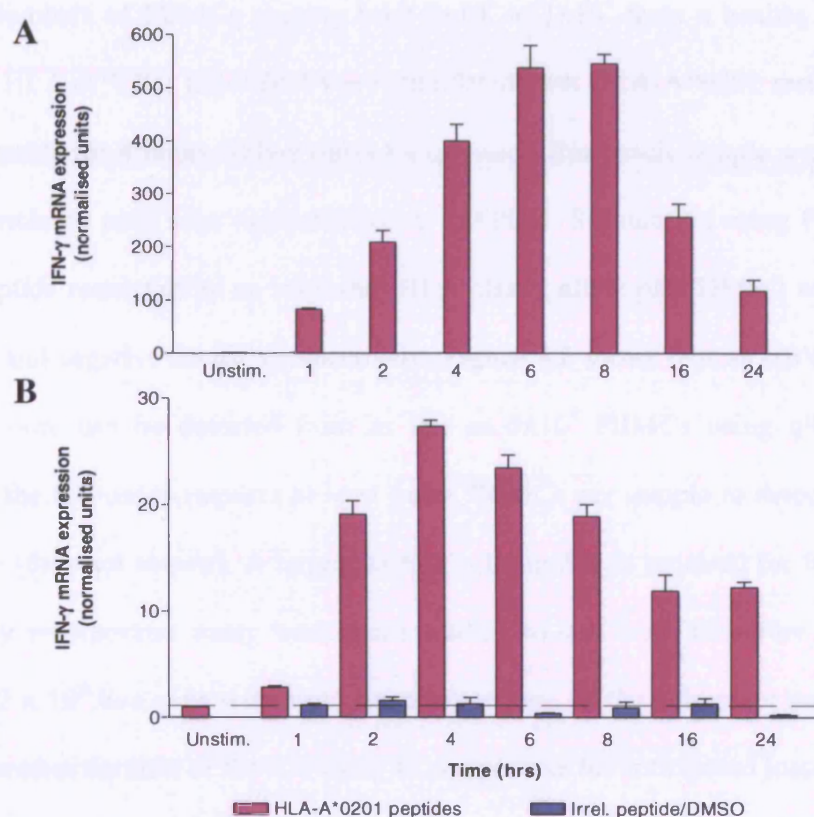


Figure 5.1: The kinetics of IFN- γ mRNA expression following stimulation with HLA-A*0201-restricted EBV peptides in a healthy HLA-A*0201 individual

1×10^6 PBMCs from an HLA-A*0201 EBV sero-positive individual were stimulated with PHA (**Panel A**) or HLA-A*0201 restricted EBV peptide pools (**Panel B**) for different periods of time. After incubation, cells were harvested, and lysed. RNA extraction, generation of cDNA and qRT-PCR for IFN- γ and GAPDH were performed as described in Chapter 2. Results are represented as IFN- γ mRNA expression relative to unstimulated cells (unstim), after normalisation to corresponding GAPDH mRNA expression from the same sample, using the $\Delta\Delta C_t$ method. Samples were performed in triplicate and presented as the mean totals. Error bars represent the sd.

5.2.1.2 Assessment of the minimum number of PBMCs required to detect IFN- γ mRNA expression in EBV-specific T cells

Experiments were performed to determine the minimum number of PBMCs from which IFN- γ mRNA expression could be detected after stimulation with EBV peptide pools. Numbers of PBMCs ranging from 1×10^6 to 1×10^4 from a healthy EBV seropositive HLA-A*0201 individual were stimulated with HLA-A*0201 restricted EBV peptide pools for 4 hours. IFN- γ mRNA expression from each sample was compared to unstimulated cells after normalisation to GAPDH. Stimulation using PHA and an EBV peptide restricted by an irrelevant HLA class I allele plus DMSO were used as positive and negative controls respectively. **Figure 5.2** shows that an EBV-specific T cell response can be detected from as few as 5×10^4 PBMCs using qRT-PCR. In contrast the ICS assay requires at least 5×10^5 PBMCs per sample to detect a positive response (data not shown). A larger starting cell number is required for ICS because the assay incorporates many wash steps leading to cell loss. Therefore in order to acquire 2×10^5 live cells within the lymphocyte gate on the cytometer more PBMCs are required at the start of the ICS assay to compensate for anticipated losses.

This result confirms the ability of qRT-PCR to detect cytokine expression from limited numbers of cells. All subsequent experiments were performed using 1×10^5 PBMCs.

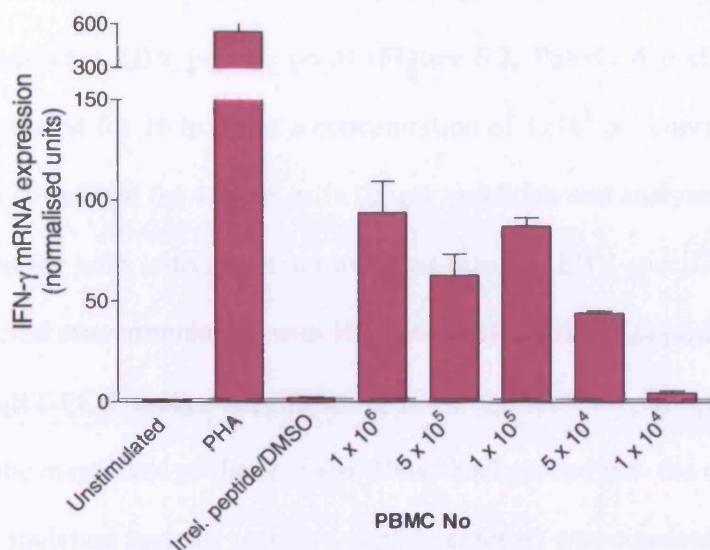


Figure 5.2: Assessment of the minimum number of PBMCs required to detect IFN- γ mRNA expression following stimulation with HLA-A*0201 restricted EBV peptide pools

Decreasing numbers of PBMCs from an A*0201 healthy individual were stimulated for 4 hours with HLA-A*0201 restricted EBV peptide pools. After incubation, cells were harvested, and lysed. RNA extraction, generation of cDNA and qRT-PCR for IFN- γ and GAPDH were performed as described in chapter 2. Results are represented as IFN- γ mRNA expression relative to unstimulated cells (unstim), after normalisation to corresponding GAPDH mRNA expression from the same sample, using the $\Delta\Delta C_t$ method. Samples were performed in triplicate and presented as the mean totals. Error bars represent the sd.

5.2.2 Comparison of ICS and qRT-PCR for measuring IFN- γ production from EBV-specific T cells after stimulation with peptides

Having established the optimal conditions for detecting a positive EBV-specific T cell response after stimulating PBMCs from healthy individuals with HLA class I-restricted EBV peptides by quantitation of cytokine mRNA expression using qRT-PCR, it was important to determine whether results obtained by qRT-PCR correlated with those obtained by ICS.

PBMCs from two healthy EBV sero-positive individuals were stimulated with HLA-A*0201 restricted EBV peptide pools (**Figure 5.3, Panels A and B**). PBMCs were either stimulated for 16 hours at a concentration of 1×10^6 per condition and analysed by ICS, or stimulated for 4 hours at 1×10^5 per condition and analysed by qRT-PCR.

The results for both individuals showed that positive EBV-specific T cell responses were detected after stimulation with HLA-A*0201 restricted peptide pools using both ICS and qRT-PCR assays despite using fewer PBMCs for the qRT-PCR assay. In addition, the magnitude of the response above background (i.e. the difference between peptide stimulation and the negative peptide control) was consistently greater in the qRT-PCR assay compared to the ICS assay (35-fold increase by qRT-PCR compared to a 6-fold increase with ICS in individual A, and 69-fold increase by qRT-PCR compared to 4-fold increase by ICS in individual B).

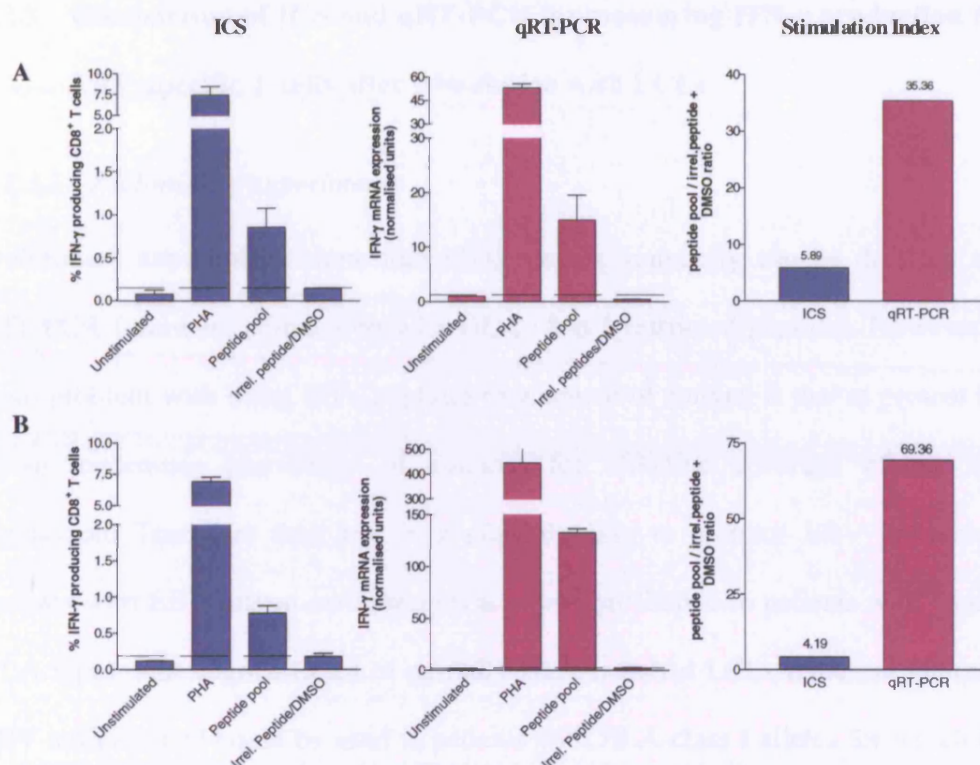


Figure 5.3: Comparison of ICS and qRT-PCR to measure IFN- γ production by EBV-specific T cells using EBV peptides as stimulators

PBMCs from two healthy A*0201 individuals (**Panel A and B**) were stimulated with an HLA-A*0201 restricted EBV peptide pool. PBMCs were either stimulated for 16 hours in the presence of Brefeldin A and analysed by ICS (■), or stimulated for 4 hours (■) and analysed by qRT-PCR. 1×10^6 PBMCs were used per condition for ICS and 1×10^5 PBMCs were used per condition for qRT-PCR. Stimulation using an EBV peptide restricted to an irrelevant HLA class I allele plus DMSO (irrelevant peptide) and PHA were used as negative and positive controls respectively. Results for ICS were expressed as percentage IFN- γ producing CD8⁺ T cells. Results for qRT-PCR were represented as IFN- γ mRNA expression relative to unstimulated cells, after normalisation to GAPDH mRNA expression from the same sample. The stimulation index was calculated by dividing the results obtained by FACs or qRT-PCR for the test sample (EBV peptide) with negative control (irrelevant peptide). Samples were performed in triplicate and presented as the mean totals. Error bars represent the sd.

5.2.3 Comparison of ICS and qRT-PCR for measuring IFN- γ production from EBV-specific T cells after stimulation with LCLs

5.2.3.1 Preliminary experiments

Preliminary experiments show that EBV specific immunity can be detected using qRT-PCR following stimulation with HLA class I restricted peptides. However, the main problem with using EBV peptides as a source of antigen is that at present there is an inadequate knowledge of epitopes for effective coverage of the entire population. Therefore their use in a clinical assay to monitor EBV immunity in patients with EBV-driven malignancies is at present limited to patients with common HLA types. Autologous LCLs or partially HLA-matched LCLs represent alternative EBV antigens and could be used in patients with HLA class I alleles for which EBV peptide epitopes have yet to be identified. Therefore to develop a qRT-PCR assay that has a broader potential for clinical application it was important to investigate whether IFN- γ mRNA expression could be detected following stimulation with autologous or partially HLA class I matched LCLs.

PBMCs from two healthy EBV sero-positive individuals were stimulated with autologous LCLs or HLA-mismatched LCLs (**Figure 5.4, Panels A and B**) at a ratio of 1:1. PBMCs were either stimulated for 16 hours at a concentration of 1×10^6 per condition and analysed by ICS, or stimulated for 4 hours at 1×10^5 per condition and analysed by qRT-PCR. IFN- γ mRNA expression was normalised to GAPDH with each test sample compared to the sample stimulated with an HLA mismatched LCLs (negative control). Amplification curves for the internal reference gene GAPDH were identical for both the test sample and negative control sample as anticipated because

input numbers of PBMCs and LCLs were the same. Therefore any increase in expression of IFN- γ induced by stimulation with autologous LCLs relative to mismatched LCLs could be assessed by comparison of IFN- γ to expression of GAPDH which was constant in both samples. Unstimulated PBMCs alone could not be used as the comparator for experiments involving stimulation with LCLs because input numbers of cells (and therefore GAPDH levels) would be different.

The results in **Figure 5.4** show that where the frequency of IFN- γ producing CD8⁺ T cells detected by ICS was relatively high (4.5%, **Panel A**) the qRT-PCR assay was able to detect a clear positive response using autologous LCLs as stimulators and produced a greater stimulation ratio (differential between response obtained by stimulation with autologous LCLs and negative LCL control) than ICS (38.4 qRT-PCR vs 19.3 ICS). However, where the frequency of IFN- γ producing CD8⁺ T cells detected by ICS was lower (1.5%, **Panel B**) the qRT-PCR performed less well.

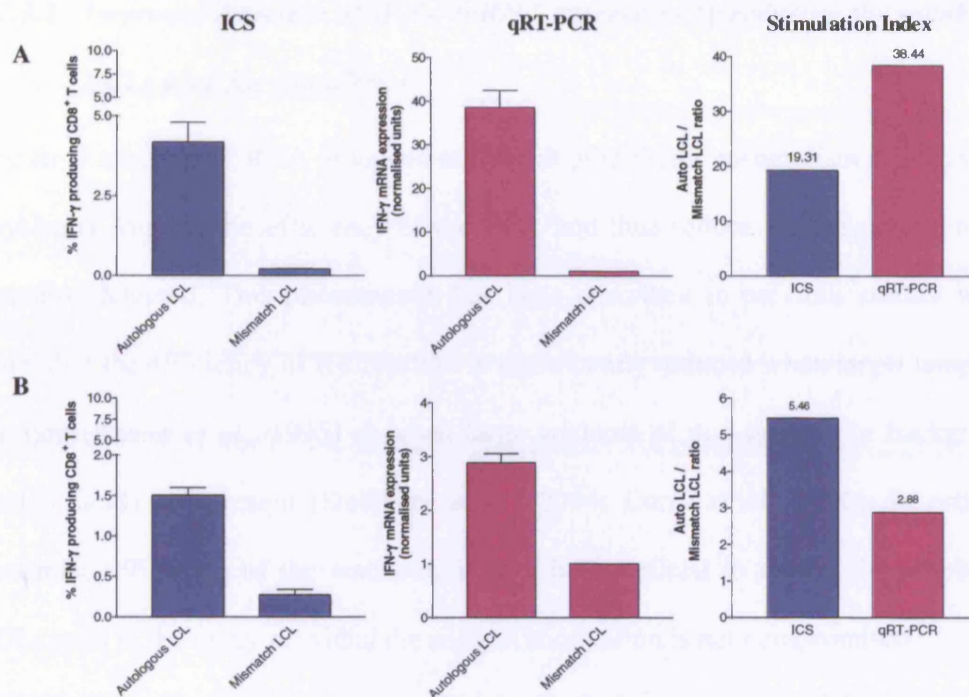


Figure 5.4: Comparison of ICS and qRT-PCR to measure IFN- γ production by EBV-specific T cells using EBV-transformed LCLs as stimulators

PBMCs from two healthy A*0201 individual (**Panel A** and **B**) were stimulated with EBV-transformed autologous LCLs (test sample). Results were compared to an HLA class I mismatched LCL (negative control). PBMCs were either stimulated for 16 hours in the presence of Brefeldin A and analysed by ICS (■), or stimulated for 4 hours (■) and analysed by qRT-PCR. 1×10^6 PBMCs were used per condition for ICS and 1×10^5 PBMCs were used per condition for qRT-PCR. Results for ICS were represented as the percentage of IFN- γ producing CD8⁺ T cells. Results for qRT-PCR were represented as IFN- γ mRNA expression relative to HLA class I mismatched LCLs after normalisation to GAPDH mRNA expression from the same sample. The stimulation index was calculated by dividing the results obtained by FACs or qRT-PCR for the test sample (autologous LCL) with negative control (HLA class I mismatched LCL). Samples were performed in triplicate and presented as the mean totals. Error bars represent the sd.

5.2.3.2 *Improved detection of IFN- γ mRNA expression by reducing the number of LCLs used for stimulation*

The large amounts of RNA produced as a result of the rapid metabolism of LCLs may physically impede the efficiency of the PCR and thus reduce the magnitude of the response detected. This phenomenon has been described in previous studies which show that the efficiency of RT reaction is significantly reduced when target templates are rare (Karrer *et al.*, 1995) or when large amounts of non-specific or background nucleic acids are present (Stahlberg *et al.*, 2004; Curry *et al.*, 2002). In order to maximise efficiency of the reactions, it may be beneficial to reduce the number of LCLs used in the assay provided the antigen stimulation is not compromised.

PBMCs from a healthy EBV sero-positive individual were stimulated for 4 hours with autologous LCLs or HLA mismatched LCLs at various PBMC to LCL ratios. The number of PBMCs was kept constant at 1×10^5 . At each ratio IFN- γ mRNA expression relative to GAPDH from PBMCs stimulated by autologous LCLs (test sample) was compared to that from PBMCs stimulated by HLA-mismatched LCLs (negative control sample). For example at the 20:1 PBMC:LCL ratio, 1×10^5 PBMCs were stimulated with either 5×10^3 autologous or 5×10^3 HLA-mismatched LCLs. IFN- γ mRNA expression is presented as the ratio of autologous LCL stimulation: HLA-mismatch LCL stimulation (**Figure 5.5**).

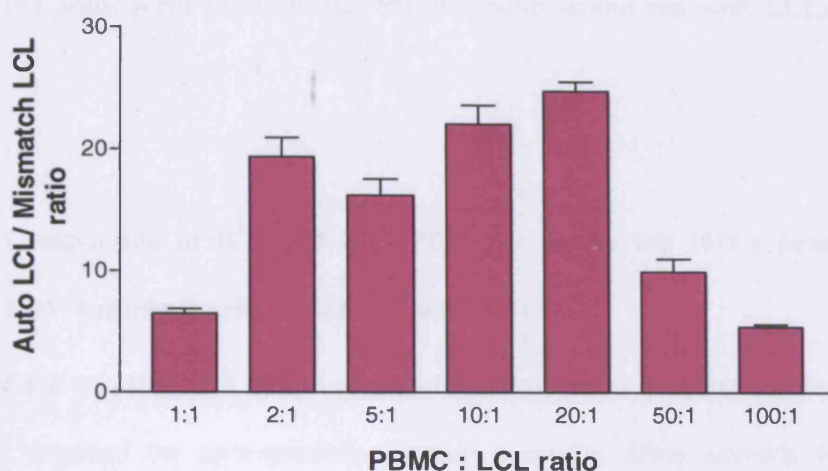


Figure 5.5: Comparison of IFN- γ mRNA expression detected from EBV-specific T cells using different numbers of LCLs as stimulators

PBMCs from a healthy EBV sero-positive individual were stimulated for 4 hours with various ratios of autologous (test) or HLA class I mismatched LCLs (negative control). The number of PBMCs was kept constant at 1×10^5 per condition. After stimulation, cells were harvested and lysed. RNA extraction, generation of cDNA and qRT-PCR for IFN- γ and GAPDH mRNA expression was performed as described in Chapter 2. At each ratio IFN- γ mRNA expression normalised to GAPDH is represented relative to the HLA-mismatch negative LCL control. All samples were performed in triplicate. Error bars represent the sd.

The results shown in **Figure 5.5** demonstrate that the qRT-PCR assay was able to detect a positive EBV-specific response from stimulation with autologous LCLs at all responder: stimulator ratios tested. The largest signal was obtained from stimulating at 20:1 ratio, with similar results obtained from ratios of 2:1 and 10:1. The signal using a 1:1 ratio however was significantly lower, consistent with inefficient PCR amplification at high template concentrations. The reduced signal at the 50:1 and 100:1 ratios is likely due to inadequate antigen stimulation with the lower numbers of

LCLs. For subsequent experiments, PBMCs were stimulated with LCLs at a 20:1 ratio.

5.2.4 Comparison of ICS and qRT-PCR for measuring IFN- γ production by EBV-specific T cells in patients with PTLD

A drawback to using ICS as the platform for analysis is that the number of viable PBMCs required for appropriately controlled results often exceeds the number available from patient samples. qRT-PCR can detect EBV-specific T cell responses from healthy individuals using 10-fold fewer PBMCs than required for ICS analysis (**Figure 5.2**). Therefore, in order to establish whether qRT-PCR could provide an alternative means of measuring EBV-specific T cell responses in patients with EBV-driven malignancies, the responses obtained from ICS were compared to those obtained by qRT-PCR.

Samples from two PTLD patients were analysed by both methods. Patient EBV⁺ 5 (**Chapter 4, section 4.1.3**) developed an EBV-positive PTLD and was treated with RIS and infusion of EBV-specific T cells. The sample used to compare the assays was collected 2.6 months after diagnosis at a time point where EBV-specific T cell immunity was rising rapidly in response to therapy (**Figure 5.6, Panel A**). Patient EBV⁺ 4 (**Chapter 4, section 4.1.3**) also developed an EBV-positive PTLD and responded clinically to treatment with RIS alone. The PBMC sample used to compare ICS with qRT-PCR was collected 9.6 months after diagnosis. At this time point a clinical response to RIS treatment had been achieved and the EBV-specific response

detected by ICS in response to both EBV peptide and autologous LCL stimulation had begun to decline in response to tumour control (**Figure 5.7, Panel A**).

PBMCs from both patients were stimulated with HLA-restricted EBV peptide pools or autologous LCLs and compared with either an EBV peptide restricted through an irrelevant HLA allele plus DMSO or an HLA-mismatched LCL respectively. The ICS assay used 1×10^6 PBMCs per condition and LCLs at a 1:1 responder to stimulator ratio whereas the qRT-PCR assay used 1×10^5 PBMCs per condition and LCLs at a 20:1 ratio. EBV-specific T cell responses from both ICS and qRT-PCR were represented as a stimulation ratio of a specific response compared to the relevant negative control.

Results showed that the qRT-PCR assay was able to demonstrate a positive EBV-specific T cell response above background in both patients despite using 10x fewer PBMCs than the ICS assay. This was achieved both in a patient where there were high numbers of circulating EBV-specific T cells detected by ICS (Patient EBV⁺ 5, **Figure 5.6**) and also in the more challenging situation of a patient whose EBV immunity had fallen to low levels in response to tumour control (Patient EBV⁺ 4, **Figure 5.7**). Comparison of stimulation ratios, however, showed that in both patients the differentiation of a positive EBV-specific response from background was better with ICS.

These preliminary results therefore suggest that qRT-PCR can be used to detect an EBV-specific T cell response in patients with PTLD using low numbers of cells.

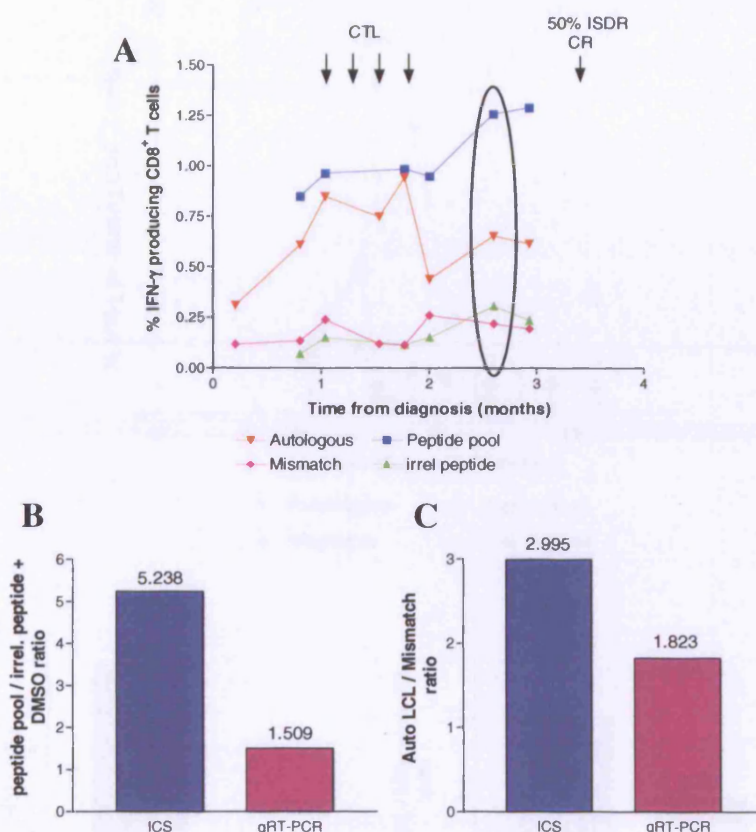


Figure 5.6: Comparison of ICS with qRT-PCR for measuring EBV-specific T cell immunity in a PTLD patient with high numbers of IFN- γ producing EBV-specific T cells

PBMCs from a PTLD patient (EBV⁺ 5) being treated with RIS and infusion of EBV-specific T cells were taken 2.6 months after diagnosis and analysed for an EBV-specific T cell response using ICS (■) and qRT-PCR (■). Serial EBV responses throughout treatment measured by ICS are shown in **Panel A**. 1×10^5 PBMCs were stimulated for 4 hours and analysed by qRT-PCR and 1×10^6 PBMCs were stimulated for 16 hours and analysed by ICS. **Panel B** shows results from PBMCs stimulated with a pool of HLA class I-restricted EBV peptides. Stimulation with an EBV peptide restricted to an irrelevant HLA class I allele plus DMSO (irrelevant peptide) was used as the negative control. Results are represented as a ratio of HLA class I-restricted EBV peptide stimulation compared to an EBV peptide restricted through an irrelevant HLA class I allele plus DMSO (peptide pool/Irrel peptide + DMSO ratio). **Panel C** shows results using PBMCs stimulated with autologous LCLs. Stimulation with an HLA-mismatched LCL was used as the negative control. LCLs were used at a 20:1 and a 1:1 responder to stimulator ratio for qRT-PCR and ICS respectively. Results are represented as a ratio of autologous LCL stimulation compared to HLA-mismatched LCL stimulation (auto LCL/Mismatch LCL ratio).

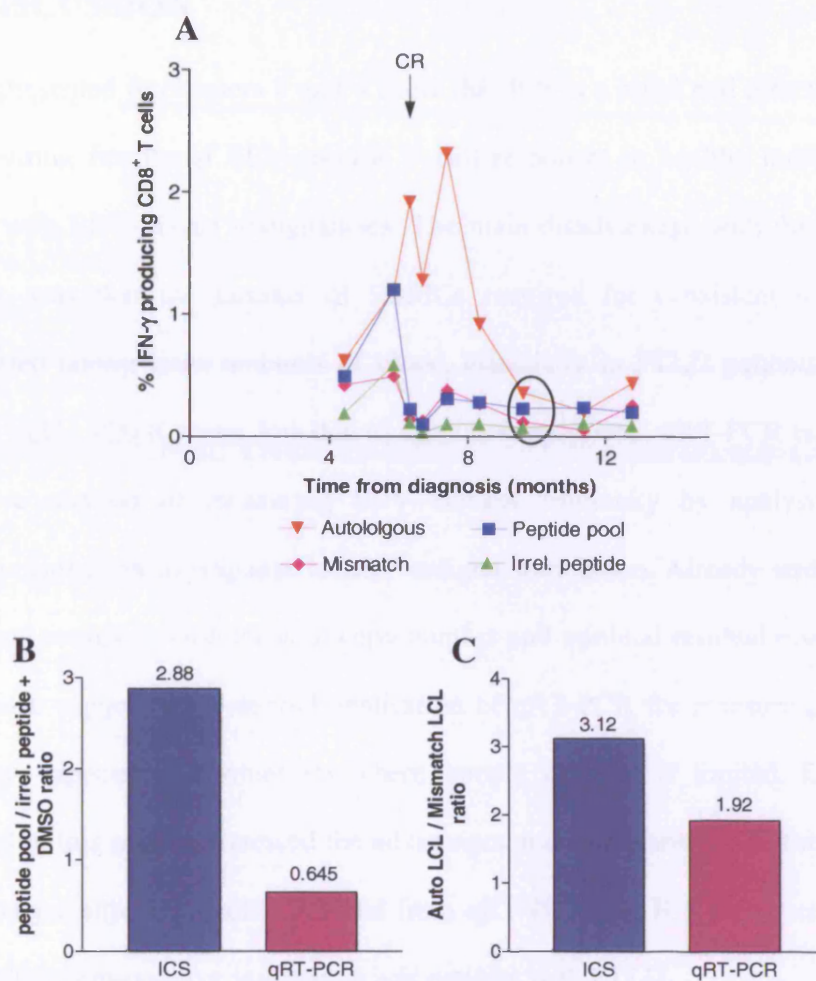


Figure 5.7: Comparison of ICS with qRT-PCR for measuring EBV-specific T cell immunity in a PTLD patient with low numbers of IFN- γ producing EBV-specific T cells

PBMCs from a PTLD patient (EBV⁺ 4) treated with RIS were taken 9.6 months after diagnosis and analysed for an EBV-specific T cell response using ICS (■) and qRT-PCR (■). See Legend 5.6

5.3 DISCUSSION

Results presented in chapters 3 and 4 show that ICS is a rapid and effective method for measuring functional EBV-specific T cell responses in healthy individuals and patients with EBV-driven malignancies. The main disadvantage with this technique, however, was that the number of PBMCs required for consistent results often necessitated taking large amounts of blood, especially in PTLD patients where the yield of viable PBMCs was low due to immunosuppression. qRT-PCR represents an alternative method of measuring EBV cellular immunity by analysing mRNA cytokine expression in response to EBV antigen stimulation. Already widely used in the clinical setting to monitor viral copy number and minimal residual disease, recent studies also suggest the potential application of qRT-PCR for measuring functional immunity, especially in situations where sample material is limited. Experiments presented in this chapter assessed the advantages and disadvantages of this technique and directly compared results obtained from qRT-PCR and ICS using samples from healthy EBV sero-positive individuals and patients with PTLD.

Functional EBV-specific T cell immunity was shown to be reliably detected using qRT-PCR from 10-fold less PBMCs than required for ICS analysis in both healthy individuals and patients with PTLD and highlights the attraction of this method in situations where samples are limited in the number of viable cells. An important consideration however, is that the qRT-PCR assay detected IFN- γ production from all cells within the PBMC sample, whereas the ICS assay was able to target IFN- γ production from CD3⁺CD8⁺ T cells within the PBMC sample using the gating strategies described in Chapter 2. In order to target this cell subset using the qRT-PCR

assay, it would have been necessary to purify CD3⁺CD8⁺ T cells from the PBMC sample prior to the stimulation experiments. Cell losses are expected with any purification process and therefore had this approach been used for the qRT-PCR assay it is probable that more PBMCs would have been required.

Unlike the ICS assay where each sample is entirely committed to analysis of IFN- γ production, only a fraction of the cDNA obtained from each test sample (approximately 1/100th) is used for a single qRT-PCR reaction. Both RNA and cDNA can be stored for several months and therefore a sample can be tested on multiple occasions and analysed for a wide spectrum of different genes of interest.

In patients with PTLN the EBV-specific T cell response can be effectively monitored with IFN- γ production from CD8⁺ T cells. However, assessment of several cytokines from the same sample would be useful for analysis of the EBV cellular immune response in EBV-positive malignancies such as PTLN, HD and NPC. For example, the predominance of CD4⁺ cells in the lymph nodes of EBV-positive HD suggests that a T-helper response plays a major role in the pathogenesis of this disease. In addition, the cellular immune response to EBV in HD patients is weak due to the virus displaying a limited set of weakly antigenic viral proteins (LMP-1 and 2) as well as the immunosuppressive environment created within the lymph node rendering EBV-specific T cells anergic (discussed in Chapter 4). Therefore in order to effectively monitor the cellular response to EBV in HD several cytokines from the T_H1 and the T_H2 response may need to be evaluated.

Results from Chapter 4 suggested an increased EBV-specific CD4⁺ T cell response in patients with EBV-positive HD compared to patients with EBV-negative HD and reactive lymphadenopathy measured by ICS. However, due to low cell numbers the

analysis was restricted to IFN- γ production as a measure of functionality and it was not practical to determine whether this represented an increased T_H1 response or an increased antigen-induced T regulatory response or a combination of both. The advantage of using qRT-PCR in this context is that mRNA expression of multiple cytokines could be evaluated from the same sample without the need for large amounts of patient material.

Initial qRT-PCR experiments showed that maximal mRNA expression of IFN- γ occurred after PBMCs had been stimulated with HLA class I-restricted EBV peptide pools for only 4 hours. RNA extraction, generation of cDNA and analysis of IFN- γ mRNA expression takes approximately 4-6 hours and therefore results for PTLD patients can be analysed and reported back to the clinician within 8-10 hours of receiving the blood sample. An overnight (16 hour) antigen stimulation period was chosen for the ICS assay for practical considerations; however this can be effectively reduced to 6 hours (data not shown). The assay then requires approximately 2 hours to acquire and analyse IFN- γ production from stimulated T cells. The turnaround time to process a sample for both assays is therefore equivalent. qRT-PCR however, offers the advantage that test samples do not need to be analysed fresh once the PBMCs have been stimulated, and can be lysed and stored at -80°C for several months.

Previous studies have demonstrated that IFN- γ mRNA transcript expression measured by qRT-PCR are good indicators of antigen T cell specificity by showing strong correlation with IFN- γ protein production from antigen-specific cells (Mocellin *et al.*, 2003; Nielsen *et al.*, 2000; Ekerfelt *et al.*, 2002; Rentzsch *et al.*, 2003; Rezvani *et al.*, 2003). Similar comparisons have not been performed using EBV antigens and it was therefore important to compare IFN- γ mRNA expression from PBMCs stimulated

with HLA-restricted EBV peptide pools and autologous LCLs obtained by qRT-PCR analysis with the frequency of CD8⁺IFN- γ ⁺ T cells determined by ICS analysis.

Results from the preliminary comparisons between the two methods of analysing the EBV-specific T cell response showed that the results from qRT-PCR analysis consistently correlated with the results obtained from ICS in both healthy individuals and patients with PTLD. Results however did not show a consistent advantage with qRT-PCR in terms of differentiating a positive EBV-specific T cell response from background compared to ICS. Differentiation of a positive response from background by the qRT-PCR assay compared to ICS was larger in healthy individuals but smaller in the PTLD patients. Reasons for this are unclear and require further investigation with serial comparisons of both qRT-PCR and ICS using healthy individuals and PTLD patients.

In conclusion, results from this chapter demonstrate that qRT-PCR can be used to detect functional EBV-specific T cells in healthy individuals and patients with PTLD. The advantages of the qRT-PCR technique are the ability to accurately detect a response from samples where cell numbers are limited, and the option to test for multiple cytokines involved in the EBV cellular immune response for each sample. A disadvantage however is that qRT-PCR instrumentation and the technical skills required are not common in clinical immunology laboratories as yet. However qRT-PCR is a clinical tool that is increasingly being used in virology and haematology laboratories. Therefore qRT-PCR may represent the future for monitoring T cell mediated antigen- specific immunity.

Chapter 6

Detection of all patterns of EBV latency in human cells and tissues using a novel monoclonal antibody, RFD3.

6.1 INTRODUCTION

The work described in the preceding chapters showed how functional CD8⁺ T cell immunity to EBV can be assessed in both healthy individuals and patients with EBV-driven malignancies, and how the response to treatment varied according to whether the tumour expressed EBV antigens. In patients with PTLD the presence of EBV antigens stimulated an EBV-specific CD8⁺ T cell response which increased as immunosuppression was withdrawn. This response was also shown to correlate with a clinical response to treatment. In contrast, although EBV immunity was detected in patients with EBV-negative PTLD tumours, no correlation with clinical response was observed. Accurate assessment of the presence of EBV antigens on PTLD tumours can therefore provide important prognostic information and influence clinical management.

In EBV-transformed LCLs and EBV-driven malignancies the virus is present latently and is transcriptionally active. Three defined types of latency have been described in which varying degrees of expression of the latent genes occur (**Table 1.2**). Latency III occurs in IM with expression of many EBV genes (EBNA-LP, EBNA2, 3A, B, C). Latency II shows a more restricted expression (EBNA1, LMP1 and 2) in malignancies such as Hodgkin's disease or nasopharyngeal carcinoma and Latency I expression is restricted to EBNA 1 occurring in Burkitt's lymphoma (Klein, 1989). This variability in expression of EBV antigens causes problems in detecting EBV in pathological samples of malignant disease and makes diagnosis difficult.

For formalin-fixed and paraffin embedded tissues an *in-situ* hybridisation technique has to be used to detect EBV encoded RNAs (EBER1 and 2) which are the most widely expressed antigens present in all stages of Latency (Howe and Steitz, 1986).

On fresh tissues, staining of EBNA1 can only be achieved by immunofluorescence or immunoperoxidase staining using immune sera from EBV seropositive individuals and employing complement fixation to amplify the signal sufficiently to detect all stages of Latency (Ohno *et al.*, 1977). Established monoclonal antibodies specific for LMP 1 and 2, EBNA 2 and BFLZ 1 are available to detect EBV but these are generally inadequate to identify all types of EBV related malignancies and frequently underestimate or are negative for malignancies that stain positively for EBER or EBNA1 (Thomas *et al.*, 1995). There is currently no monoclonal antibody which is capable of identifying all EBV positive transformed cells. Monoclonal antibodies to EBNA 1 have been described but these have failed to reproduce results reliably enough to support commercial development (Grasser *et al.*, 1994; Oudejans *et al.*, 1996).

During immunohistological staining of fresh frozen tissues, nuclear reactivity of a murine monoclonal antibody, RFD3, was observed in EBV-driven lymphoproliferation. RFD3 is an IgG2 antibody that stains follicular dendritic cells (FDCs) and collagen (particularly associated with basement membranes) strongly and mesangial cells weakly (Bofill *et al.*, 2000).

The aim of the work described in this chapter was to explore whether RFD3 represents a reagent capable of detecting all patterns of EBV latency. IP staining using RFD3 was carried out on a range of EBV-positive and -negative cell lines and tissues, and compared to current methods of EBV detection. Two HHV-8-positive, EBV-negative cell lines, one CMV-positive, EBV-negative cell line and malignant tissues were investigated to determine the cross-reactivity of RFD3 with other herpes viruses. In addition the efficacy of RFD3 in detecting EBV in cytopins and fresh or formaldehyde-fixed tissues was assessed.

6.2 RESULTS

6.2.1 Nuclear staining of fresh frozen cryostat sections of lymph node and tumour tissues by RFD3.

The monoclonal antibody RFD3 is known to bind to unidentified collagen-like material which is expressed on mature follicular dendritic cells and identifies networks within lymphoid follicles and germinal centres (Bofill *et al.*, 2000). RFD3 also binds to the basement membranes of blood vessels and intercellular material that is markedly accentuated in fibrotic conditions. In addition to this staining pattern, it was observed that speckled nuclear staining occurred occasionally in pathological tissues of EBV-driven malignancies. Examples are shown in **Figure 6.1**.

In order to examine the association between the presence of RFD3 nuclear staining and the EBV status of a tumour, several non-malignant reactive LNs and malignant B cell lymphoma (ML-B) LN sections were stained using the mAb RFD3. All samples from diseases with known EBV association were screened for presence of EBV by EBER *in situ* hybridisation (EBER ISH) or EBNA Complement Fixation staining (EBNA-CFT) to confirm their EBV status. Formal identification of EBV negative status was performed for representative samples of the diseases that are not known to be associated with EBV. The results are summarised in **Table 6.1** and show a strong agreement between EBV and RFD3 nuclear staining. Of note, tissues from 2 patients with ML-B: diffuse large cell type (ML-B:DLC) stained positive with RFD3, a result which was confirmed by EBNA-CFT analysis. This disease is not typically associated with EBV; however both ML-B DLC samples that stained

positive with RFD3 were from patients that also had coexisting immunodeficiencies that are known to confer an increased risk of EBV associated lymphomas.

Table 6.2 details the percentage of positive nuclei in EBV positive pathologies as examined by the three methods of EBV detection. Statistical analysis of this table showed a strong correlation between RFD3 nuclear staining and EBER-ISH staining ($r = 0.909$) or EBNA-CFT ($r = 0.937$). Correlation coefficients were calculated using the Pearson's method.

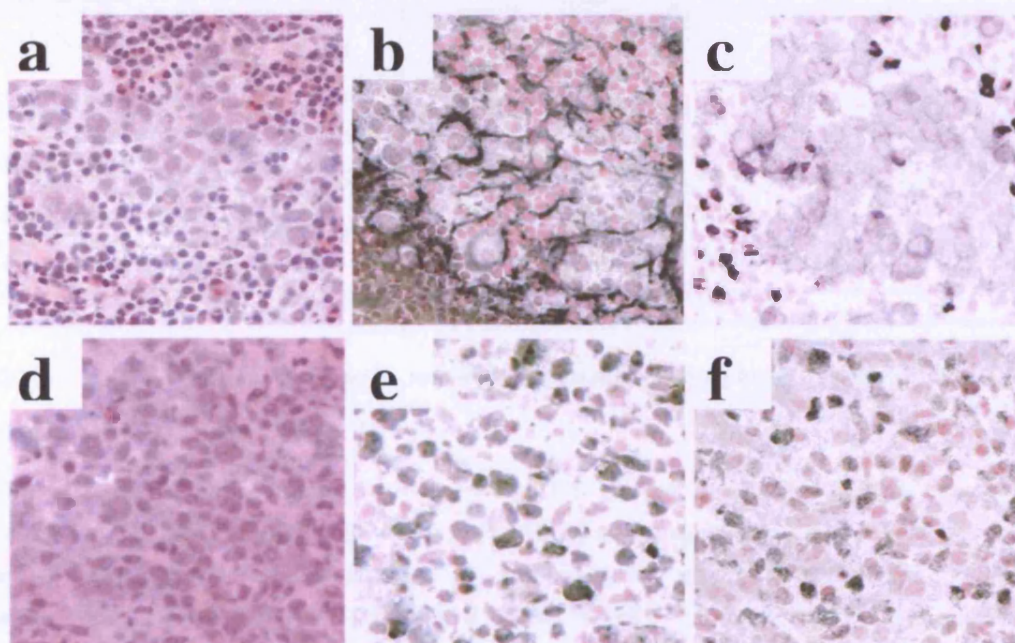


Figure 6.1: RFD3 nuclear staining of EBV-positive tissues

EBV-positive HD (**Panels a-c**) and malignant lymphoma B cell type: diffuse large B cell (**Panels d-f**) were stained using the following techniques. Haematoxylin and Eosin (H&E) stain: **Panels a,d**; RFD3 mAb: **Panels b, e**; LMP mAb: **Panel c**; EBNA Complement Fixation staining (EBNA-CFT): **Panel f**.

Unstained nuclei are counterstained with Fast Red and show as pale pink.

<i>Disease</i>	<i>No</i>	<i>EBV status</i>	<i>Pre-ID</i>	<i>RFD3n</i>
ML-B PTLD	12	11 positive 1 nd	12	12
ML-HD	8	1 positive	0	1
ML-NK LC	1	1 positive	0	1
RH/RFH	20	3 negative 17 nd	0	0
Non-malignant reactive lymph nodes	6	2 negative 4 nd	2	0
ML-B B/BL	2	2 negative	1	0
ML-B F	16	1 negative 15 nd	0	0
ML-B MZ	4	nd	0	0
ML-B CLL	4	nd	0	0
ML-B DLC	26	17 negative 2 positive 7 nd	3	2
ML-ALC	3	1 positive	0	1
Kaposi sarcoma	3	3 negative	3	0
Carcinoma	2	nd	0	0

Table 6.1: RFD3 nuclear staining in reactive, malignant and lymphoma tissues in relation to EBV associated pathologies

No: Number of lymph nodes or tissues examined. **EBV status:** Presence of EBV in tissue was assessed by EBER or EBNA CFT staining. **Pre-ID:** Pre-existing immunodeficiency or intensive immunosuppression. Two patients in the non-malignant group had Common Variable Immunodeficiency (CVID) and 1 ML-B: B/BL patient had HIV. All 12 ML-B PTLD patients were on long-term immunosuppression for solid organ transplants and all patients with Kaposi's sarcoma were immunosuppressed due to HIV. Of the 26 ML-B: DLC patients, 3 had immunodeficiency; 1 secondary to CVID, 1 secondary to HIV and 1 secondary to chronic active hepatitis. **RFD3n:** Number of samples positive by RFD3 nuclear staining. **RH/RFH:** Reactive hyperplasia or reactive follicular hyperplasia of unknown cause. **Non-malignant:** Castlemann's disease 1, Granulomatous disease 2, Histiocytic reaction 1, Common Variable Immunodeficiency 2. **ML-B** Malignant lymphoma B cell type: **ALC** – Anaplastic Large Cell, **B/BL** – Burkitt's or Burkitt-like, **F** - follicular, **MZ** - marginal zone, **DLC** - diffuse large cell, **CLL** - chronic lymphocytic leukaemia, **HD** - Hodgkin's disease, **NK LC** – Natural killer large cell, **PTLD** – Post transplant Lymphoproliferative Disease. **Nd:** Not done

<i>Lymphoma</i>	<i>RFD3n</i>	<i>EBER-ISH</i>	<i>EBNA-CFT</i>	<i>RFD3-FDC</i>	<i>Pre-ID</i>
ML-B ALC	10	10	10	-	0
ML-B DLC	80		50	-	CAH + HD
ML-B DLC	30		20	-	HIV
ML-HD	7	7		-	0
ML-NK LC	20	30		-	0
ML-B PTLD	30	35		-	IS/RT
ML-B PTLD	1	0	0	-	IS/RT
ML-B PLTD	30	40	35	-	IS/RT
ML-B PTLD¹	75	60	60	+	IS/RT
ML-B PTLD²	75	40	55	+	
ML-B PTLD³	7		0	-	
ML-B PTLD	35		40	-	IS/OLT
ML-B PTLD	70	60	65	-	IS/RT
ML-B PTLD	70		80	-	IS/RT
ML-B PTLD¹	15		10	+	IS/RT
ML-B PTLD²	10	10	7	+	
ML-B PTLD	12	12	12	-	IS/RT

Table 6.2: Percentage of nuclei staining by RFD3, EBER and EBNA CFT in EBV positive pathologies

Key: As for Table 1.

RFD3-FDC: Staining positive for follicular dendritic cells by RFD3

CAH + HD: Chronic active hepatitis and previous Hodgkin's disease

HIV: Human immunodeficiency virus positive

IS/RT: Immunosuppression for a renal transplant

IS/OLT: Immunosuppression for a liver transplant

Superscripts 1, 2 & 3 indicate sequential tissue biopsies on the same patient.

In order to assess whether RFD3 could be employed on formalin-fixed tissues, several EBV-positive samples were tested (Ms R Sims, department of Histopathology, Royal Free Hospital). Despite trialling a range of preparation techniques prior to staining (See **Materials and Methods 2.5.3**) all results were negative (data not shown), confirming the use of RFD3 in detecting EBV in fresh tissue samples only.

6.2.2 Comparison of the efficacy of RFD3 versus LMP/EBNA-2 (IP) and EBNA CFT staining, using a range of EBV positive and negative cell lines

To confirm the immuno-histological findings using tissue that RFD3 stains nuclei of EBV positive cells, cell lines of known EBV status were examined using the monoclonal antibodies RFD3, anti-EBNA-2, anti-LMP-1 and -2 and EBNA-CFT. Only nuclear staining was evaluated. Some cell lines gave variable amounts of cytoplasmic staining, particularly with EBNA-CFT. Since EBNA-CFT uses EBV positive human sera as a source of anti-EBNA1 antibody, cytoplasmic staining is often found and is not of EBV-related significance.

Figure 6.2 shows the pattern of staining obtained from EBV-positive cell lines and shows consistent nuclear staining between all three mAbs tested.

The proportion of cells correctly identified by nuclear staining from RFD3, anti-EBNA-2 and anti-LMP was analysed and compared to results obtained by EBNA-CFT (**Table 6.3**). RFD3 was effective in identifying EBV positive cell lines and there was concordance with EBNA-CFT staining on all human EBV-transformed LCLs except for LCL 4 where only a low percentage of cells was detected by RFD3. The number of cells stained positive by RFD3 was similar or a little above the

proportion stained by anti-EBNA-2 mAb using the IP method and always more than the number stained positive by anti-LMP mAbs. When staining EBV-positive human Burkitt's lymphoma cell lines, EBNA-CFT and IP staining with RFD3 yielded similar results, with the proportion of cells stained positive always higher than those detected using anti-EBNA-2 and anti-LMP mAbs. This was also the case with the EBV-positive acute lymphoblastic leukaemia (ALL) cell line, HPB-Null and the unidentified EBV-positive cell line Ramal. RFD3 however, detected a much higher level of positivity (90%) in a second ALL cell line, KM-3 compared to detection by EBNA-CFT (4%).

Overall correlations between the positive results obtained by RFD3 nuclear staining compared to EBNA-CFT and EBNA2 were strong ($r=0.701$ and $r=0.795$, respectively, using the Pearson's method), confirming the ability of RFD3 to detect the presence of EBV. The correlation between RFD3 and LMP however, was less ($r=0.335$) reflecting the previously described inconsistent results obtained with established mAb staining (Thomas *et al.*, 1995).

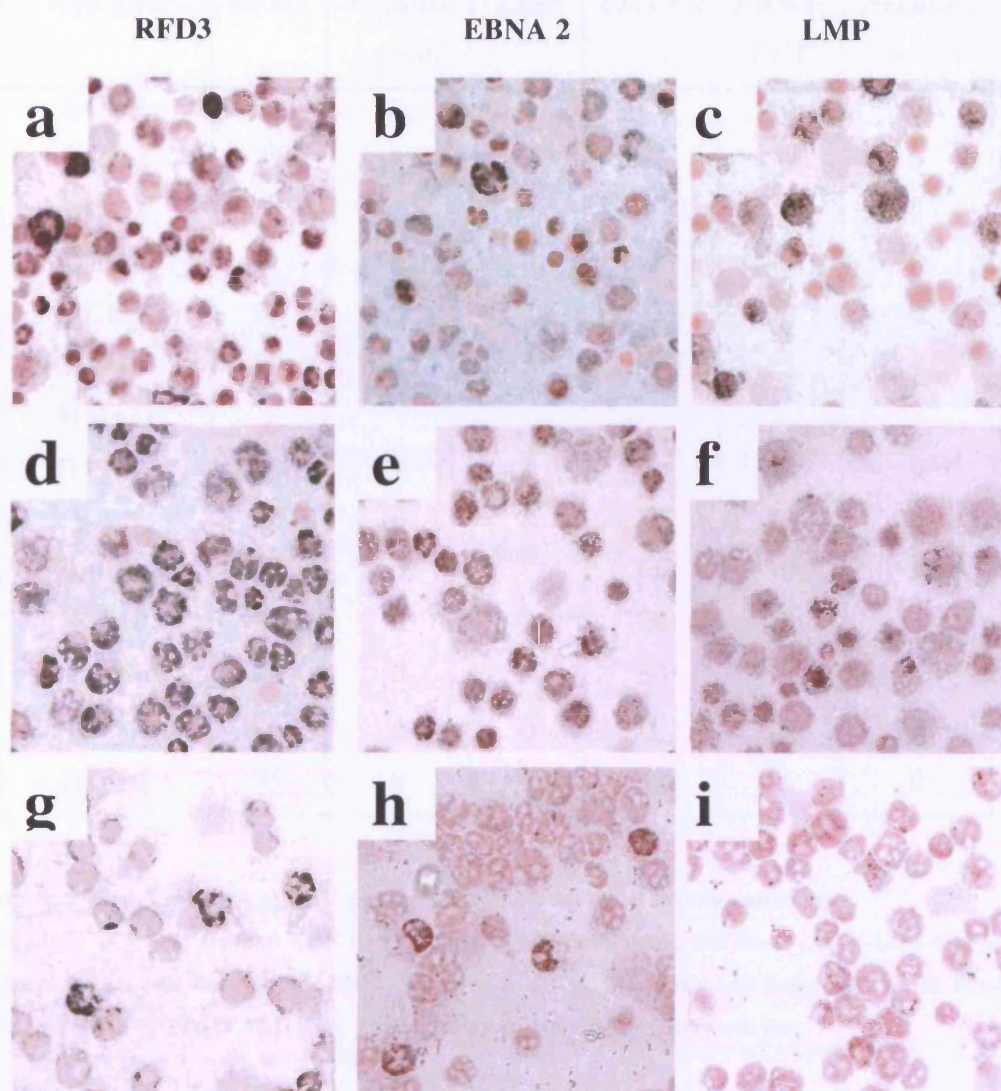


Figure 6.2: Staining of EBV-positive cell lines with RFD3, EBNA2 and LMP mAbs

EBV-positive cell lines were stained with RFD3 (Panels a, d, e), EBNA2 (Panels b, e, h) and LMP (Panels c, f, i). The cell lines used were EBV-transformed LCLs (Panels a-c) and EBV-positive Burkitt's cell lines, Raji (Panels d-f) and KM-3 (Panels g-i).

<i>Cell Line</i>	<i>RFD3</i>	<i>Negative control</i>	<i>LMP</i>	<i>EBNA-2</i>	<i>EBNA-CFT</i>	<i>Negative control</i>
LCL 1	80	0	7	75	80	0
LCL 2	75	0	60	65	80	0
LCL 3	7	0	0	0	2	0
LCL 4	5	0	0	0	90	0
LCL 5	85	0	7	75	90	0
LICR(-LON-HMy2)	90	0	7	90	95	3
DAUDI	95	1	1	5	95	0
EB-3	30	0	5	0	25	0
Namalwa	85	0	0	65	90	0
RAJI	90	0	3	70	95	0
HPB Null	90	0	15	80	90	0
KM-3	90	0	0	3	4	0
Ramal	95	0	0	40	95	0

Table 6.3: RFD3 staining of EBV-positive cell lines compared to other methods

Cell lines: LCL 1-5: Human EBV transformed B lymphoblastoid cell lines; LICR(-LON-HMy2): plasmacytoma cell line; Daudi, EB-3, Namalwa and Raji: Burkitt's cell lines; HPB Null: Pre-B ALL line; KM-3: Pro-B ALL line; Ramal: unidentified EBV-positive cell line.

Almost all EBV-negative cell lines were negative when tested with the range of antibodies used to detect EBV (**Table 6.4**). There was some cytoplasmic staining of the human acute T cell lymphoblastic leukaemia cell lines, C8166 and U937 cells and extremely weak speckled staining of the small cell lung carcinoma cell line, H69 (5-10%) using RFD3. Correspondingly, when using the EBNA-CFT method, there was cytoplasmic staining of the cell lines, C8166, CCRF-CEM, HL-60 and U937 using the anti-EBNA antibody or the mouse serum (negative control). Speckled

staining occurred in the cell lines HPB ALL and H69, however this was too weak to have led to a positive designation.

<i>Cell line</i>	<i>RFD3</i>	<i>Mouse serum</i>	<i>LMP</i>	<i>EBNA-2</i>	<i>EBNA-CFT</i>	<i>Negative control</i>
C8166	0	0	0	0	0	0
CCRF-CEM	0	0	0	0	0	0
HPB ALL	0	0	0	0	90	0
Jurkat	0	0	0	0	0	0
MOLT-3	0	0	0	0	0	0
HL60	0	0	0	0	0	0
K562	0	0	0	0	0	0
Ramos	0	0	0	0	20	40
U937	0	0	0	0	0	0
MWHDL	0	0	0	0	0	0
H69	7	0	0	0	70	0

Table 6.4: RFD3 staining of EBV-negative cell lines compared to other methods

Cell lines: C8166, CCRF-CEM, HPB-ALL, Jurkat, MOLT-3: Human acute T lymphoblastic leukaemia; HL60: human promyelocytic leukaemia; K562: human chronic myelogenous leukaemia; Ramos: Burkitt lymphoma; U937: Histiocytic cell line; MWHDL: Mesothelioma cell line; H69: human small cell lung carcinoma. Normal mouse serum was used as a negative control for the mAb staining. Human serum from an EBV sero-negative individual was used as the negative control for detection of EBV using EBNA-CFT.

6.2.3 Determining the ability of RFD3 to identify cells infected with other members of the herpes virus family, namely HHV-8 and CMV

In order to determine whether RFD3 cross-reacted with antigen from other herpes viruses, RFD3 was used to stain HHV-8 and CMV positive cell lines. Staining HHV-8-positive, EBV-negative cell lines with RFD3 gave consistently positive

results. Negative results however were seen using the LMP and EBNA2 antibodies as well as normal mouse serum.. In contrast RFD3 did not show any positive staining in the cells of a CMV-positive, EBV-negative cell line. Negative staining was also seen with the LMP and EBNA2 antibodies and the negative control (**Table 6.5**).

<i>Cell line</i>	<i>RFD3</i>	<i>Mouse serum</i>	<i>LMP</i>	<i>EBNA-2</i>	<i>LN53</i>	<i>CMV</i>	<i>EBNA-CFT</i>	<i>Negative control</i>
BC-3	95	0	0	0	50	nd	0	0
BCP-1	75	0	0	0	95	nd	0	0
BJ1	0	0	0	0	nd	90	0	0

Table 6.5: RFD3 staining of different Herpes virus positive cell lines compared to other methods

Cell lines: **BC-3 and BCP-1**: HHV-8-positive, EBV-negative cell lines; **BJ1**: CMV-positive, EBV-negative fibroblast cell line (AD169 strain). Normal mouse serum was used as a negative control for the mAb staining. Human serum from an EBV sero-negative individual was used as the negative control for detection of EBV using EBNA-CFT.

The pattern of staining of HHV-8-positive cell lines observed with RFD3 however, yielded an atypical pattern in comparison to that seen with the EBV-positive cell lines. In addition, both pattern and percentage of cells detected differed from the nuclear staining seen with the monoclonal antibody LN53, currently used to detect the latent nuclear antigen-1 (LTA-1) expressed in all HHV-8 positive cells (Dupin *et al.*, 1999). It must be noted that LN53 also appeared to detect linear structures within the nucleus of Raji (EBV-positive, HHV-8-negative) cells (**Figure 6.3**).

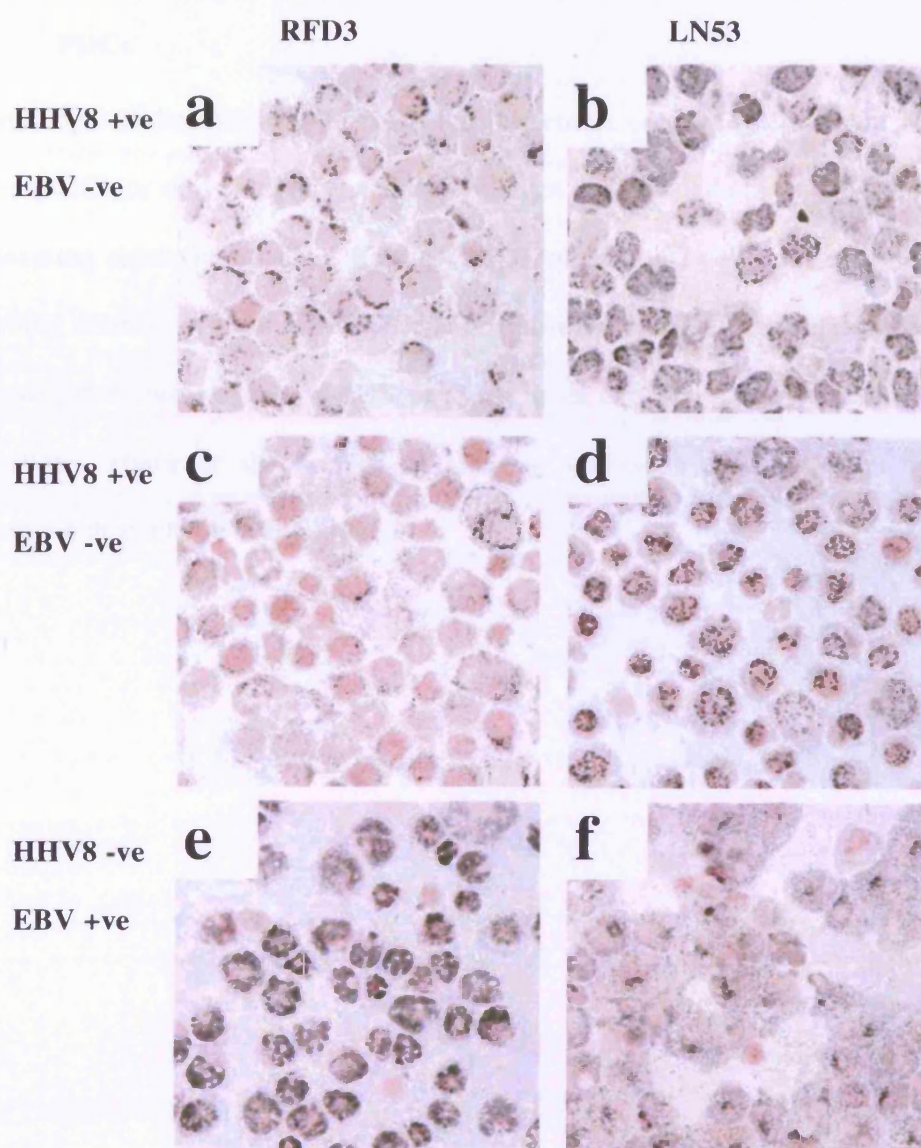


Figure 6.3: Staining of herpes virus-positive cell lines with RFD3 and LN53 mAbs

Cell lines were stained with RFD3 (Panels a, c, e), and LN53 (Panels b, d, f). The cell lines used were HHV-8-positive, EBV-negative BC3 (Panels a-b) and BCP (Panels c-d) as well as the HHV-8-negative, EBV-positive Burkitt's cell line, Raji (Panels e-f).

6.2.4 Attempting to separate staining of EBV positive nuclei and staining of FDCs

Immunoperoxidase staining of fresh tonsil sections containing follicular dendritic cells (FDC), or skin sections containing collagen, was carried out using RFD3 mAb-containing supernatants taken from 47 RFD3 monoclonal cell lines established by limiting dilution. All tissue sections stained positive for FDCs or collagen (data not shown), therefore staining of EBV-positive nuclei and FDCs/collagen could not be separated. However the pattern of staining was such that the three cellular components could be differentiated.

6.3 DISCUSSION

The detection of EBV in pathological sections of EBV-driven malignancies is currently determined by an *in situ* hybridisation technique used to identify the presence of EBV encoded RNA (EBER). The expression of EBER is seen across all latency patterns of EBV and therefore identifies all EBV-positive cells. This method however is cumbersome and only possible on formalin-fixed samples. IP staining with monoclonal antibodies offers a quicker alternative but to date antibodies to EBV antigens have produced unreliable results. In addition, a panel of antibodies is required to ensure adequate identification of EBV positive cells across all stages of EBV latency. The results presented in this chapter provide evidence that the mouse monoclonal antibody, RFD3 is able to detect an unidentified nuclear antigen present in all latency patterns of EBV-positive cells and therefore has potential as a useful diagnostic and research tool.

Of the 107 samples from patients with EBV associated pathologies, a positive EBV diagnosis as predicted by RFD3 was confirmed by EBER-ISH or EBNA-CFT in 16 samples. One PTLN sample was weakly positive with RFD3 but was determined EBV negative according to EBNA-CFT (**Tables 6.1 and 6.2**). Overall, there was strong correlation in the number of positive nuclei when RFD3 staining was compared to the current diagnostic methods used to detect EBV ($r = 0.9168$ with EBER-ISH and $r = 0.9323$ with EBNA-CFT).

To confirm and elaborate on the above-described results, 24 cell lines were investigated with the monoclonal antibodies, RFD3, anti-LMP 1, anti-LMP 2 and anti-EBNA-2. EBV was also detected using EBNA-CFT. Of the 11 assumed EBV negative cell lines, one consistently stained weakly positive with RFD3. Weakly positive staining was also observed in this cell line using EBNA-CFT. 11 of the 13

cell lines assumed to be EBV positive were confirmed unequivocally positive by RFD3 staining. Of the two cell lines that stained weakly by RFD3, one was also weakly positive by EBNA-CFT staining and the other was strongly positive (**Table 6.3**). Overall correlation between the number of EBV positive nuclei determined by EBNA-CFT and RFD3 remained high ($r = 0.701$) confirming the clinical utility of RFD3 as a diagnostic tool for detecting EBV in pathological samples.

Despite the observation that gene expression in EBV positive Burkitt's lymphoma cell lines can, with increasing passage number, be more strongly related to latency III than latency I (Gregory *et al.*, 1990), the results obtained with LMP and EBNA-2 (**Table 6.3**) suggest that at least two of the BL lines, DAUDI and EB-3, have a latency I expression pattern. Thus, the cell lines used in these experiments were representative of expression patterns associated with all three malignancy-related latency types (Klein, 1989).

The possibility that RFD3 detected other herpes virus-positive cells was investigated using HHV-8-positive and CMV-positive cell lines. Although CMV-positive, EBV-negative cells showed negative staining by RFD3, in cells of HHV-8-positive, EBV-negative lines RFD3 detected small round structures that appeared to border the nucleus (**Figure 6.3**). Likewise, there was staining of the EBV-positive, HHV-8-negative Raji cells with the established anti-HHV-8 antibody. Explanations for this include homology between the nuclear antigens present in HHV-8 and HHV-4 infected cells. However, although similarities have been described between HHV-8 genes and EBV genes encoding the nuclear antigens, LMP 1 and 2 (Glenn *et al.*, 1999) and the membrane antigen p140 (Cesarman *et al.*, 1996), no homology has been described between HHV-8 genes and the other EBV nuclear antigens expressed in different latency patterns, all of which RFD3 has been shown to

positively identify. Additional staining with RFD3 on HHV-8-positive Kaposi's sarcoma (KS) samples yielded negative results (results not shown), suggesting that RFD3 is exclusively associated with EBV in pathological tissue sections. Possible reasons for the presence of confounding staining with RFD3 in cell lines but not tissue samples include different patterns of HHV8 gene expression in the cell lines compared to the KS samples.

The results obtained thus far are insufficient to ascertain the identity of the nuclear antigen detected by RFD3 monoclonal antibody. Of the known EBV antigens, only EBNA 1 is present in all three forms of malignancy-related latency (Klein, 1989), however there is also evidence for the maintained expression of the family of alternatively spliced *Bam*HI A fragment transcripts in EBV-transformed cells (Brooks *et al.*, 1993). Three open reading frames have been investigated in detail, and there is some data suggesting the putative protein products of two of these, RPMS1 and RK-BARF0 may be nuclear (Kienzle *et al.*, 1999; Smith *et al.*, 2000; Sun *et al.*, 1998). Finally, the antigen detected could be EBV-induced of host origin. Further investigation is required to determine the antigen's function as related to EBV latency; however its presence across all latencies suggests the possibility of using RFD3 in the diagnosis of EBV-related malignancies.

Chapter 7

General Conclusions

7.1 GENERAL CONCLUSIONS

Work detailed in this thesis describes the development of a flow cytometry based assay to measure functional IFN- γ producing EBV-specific T cells, and assesses its role in the clinical management of EBV-driven malignancies.

The rationale for developing this assay was primarily directed at PTLD patients where uncontrolled EBV-driven B cell proliferation is caused by suppression of the host's natural EBV immunity secondary to immunosuppressive drugs used post transplant to control alloreactivity and prevent graft rejection. In these patients gradual withdrawal of these drugs allows immune recovery and the re-emergence of functional EBV-specific T cells which in turn leads to effective tumour control and resolution of the disease. At present the emergence of this EBV response is monitored using an immunophenotyping assay which identifies increases in activation markers such as MHC Class II and CD69 (Amlot *et al.*, 1996). Using serial data from the point of diagnosis the rate of withdrawal of immunosuppression can be tailored to each individual PTLD patient (Amlot *et al.*, 2007). Although this approach is effective, it measures the recovery of all activated T cells following RIS but does not provide information on their antigen specificity.

Measurement of IFN- γ producing CD8⁺ T cells was shown to consistently demonstrate functional EBV immunity in healthy individuals and patients with PTLD or HD. Further, in PTLD patients a temporal relationship was observed between the emergence of IFN- γ producing EBV-specific CD8⁺ T cells and regression of the EBV-associated tumours in response to RIS treatment. These findings confirm the crucial role of these cells in the elimination of malignant cells transformed by EBV and supports previous indications that IFN- γ producing CD8⁺ T cells provide protective immunity in healthy individuals (Hislop *et al.*, 2001) and

in patients with IM (Callan *et al.*, 2000; Hoshino *et al.*, 1999). IFN- γ producing CD8⁺ T cells have also been recently shown to be specific for EBNA-1, an EBV antigen previously thought not to be recognised by CD8⁺ T cells because responses could not be detected by traditional cytotoxicity assays (Lee *et al.*, 2004). IFN- γ production may therefore represent a more effective indicator of target cell recognition in the context of EBV.

Intracellular staining for cytokine production was shown to be a rapid and effective means of detecting functional EBV-specific CD8⁺ T cells and therefore amenable to routine diagnostic use. The T cell mediated immune response to EBV has been characterised in healthy individuals and patients with primary EBV infection using a variety of techniques including LDAs, cytotoxicity assays, ELIspot assays and fluorochrome-labelled HLA class I:peptide tetramers (detailed in chapter 3). However, while each of these assays is commonly used in the context of research, each has limitations prohibiting their use as a clinical assay (**Table 3.1**). ICS however, has the advantage over the above techniques of generating results rapidly as well as using a flow cytometer and thus equipment universal to all clinical immunology laboratories. Kuzushima *et al* described use of this technique to enumerate EBV-specific T cells in healthy individuals and showed superior sensitivity compared to assessment of cytotoxic T cell precursor frequencies by the LDA or detection of IFN- γ producing T cells by the ELIspot assay (Kuzushima *et al.*, 1999). The ICS assay was subsequently shown to effectively illustrate the dynamics of EBV-specific CD8⁺ T cells in a single patient with PTLD following HSCT (Kuzushima *et al.*, 2000).

Assessment of cytokine gene expression by qRT-PCR was shown to be an alternative method of detecting EBV-specific T cells in both healthy individuals and

patients with PTLD. This is the first report of use of qRT-PCR to examine EBV immunity, and preliminary results showed a consistent correlation with those obtained by ICS, supporting previous studies suggesting that IFN- γ mRNA transcript expression is an effective surrogate for cytokine production by antigen specific T cells (Ekerfelt *et al.*, 2002; Mocellin *et al.*, 2003; Nielsen *et al.*, 2000; Rentzsch *et al.*, 2003; Rezvani *et al.*, 2003). It was also shown that qRT-PCR requires fewer PBMCs to elicit a positive result, making this an attractive alternative method of detecting the cellular immune response to EBV in paediatric or severely lymphocytopenic PTLD patients where cell numbers are limited. A caveat however is that the qRT-PCR experiments performed with PBMCs did not allow identification of the cell population producing IFN- γ , whereas ICS facilitates visualization of the CD8⁺ T cells within the PBMC population and therefore targets detection to a specific cell subset. A direct comparison of cell numbers required in qRT-PCR and ICS for measurement of IFN- γ production by CD8⁺ T cells therefore requires further experiments using a cell sorting technique to isolate CD8⁺ T cells prior to analysis by qRT-PCR.

Autologous LCLs provided an excellent source of EBV antigen as they constitutively express the complete range of EBV latent cycle antigens (latency pattern type III) which is the predominant expression pattern in EBV associated PTLD. In patients where it was not possible to transform their B cells, LCLs matched on multiple HLA class I alleles were used as an effective alternative stimulator. Due to the phenomenon of immunodominance, LCLs matched for only one HLA-A or HLA-B allele did not give consistently positive results. Pools of HLA class I restricted EBV peptides were also effective at stimulating an EBV-specific CD8⁺ T cell response, but this approach is hindered by the limited number

of EBV epitopes described to date. Epitopes presented by rarer HLA class I alleles and HLA types in non-Caucasoid populations have yet to be characterised.

The emergence of IFN- γ producing CD8⁺ T cells detected by serial monitoring of PTLD patients treated with RIS mirrored the rise in MHC class II expression by CD8⁺ T cells during step-wise withdrawal of immunosuppression. This assay is used in conjunction with the disease and performance status of the patient to provide an effective and individually tailored approach to RIS and has been shown to limit the incidence of graft rejection caused by arbitrary or too rapid withdrawal of immunosuppressive drugs (Amlot *et al.*, 2007). Advantages of this technique include its speed; results can be generated within 1-2 hours of receiving a sample and its low cost; analysis of surface activation markers can be obtained with a panel of 15 monoclonal antibodies using a three-colour flow cytometer. Cost would be further reduced however, if the immunophenotyping assay was simplified to measure MHC class II surface expression on CD8⁺ T cells alone. The good correlation observed between rise in functional EBV specific T cell immunity with the increase in numbers of CD8⁺ T cells expressing surface activation markers supports the view that the immunophenotyping assay is sufficient for guiding RIS. However, this assay does not show which antigen is stimulating the rise in activated T cells and is limited to evaluating the capability of the immune response to infection in the context of RIS. In addition, changes in the frequency of CD8⁺ T cells expressing MHC class II can be seen with alloreactivity occurring in bowel transplants. This has not been demonstrated in renal or liver transplantation, however (P.Amlot, personal communication). In contrast an assessment of cytokine production in response to EBV antigen stimulation has the advantage of providing a specific measure of EBV immunity. A potential draw-back of switching from the

immunophenotyping assay to the ICS assay is that a source of EBV antigen needs to be readily available for each PTLD patient, either in the form of an autologous LCLs or a LCLs matched on multiple HLA class I alleles. Facilities for cell cryopreservation are therefore necessary. In addition, although it is assumed that PTLD tumours express the full range of EBV antigens, many studies have shown that this is an oversimplification and that EBV antigen expression is variable. Therefore, it is possible that the EBV-specific T cell response may be missed in some PTLD patients with tumours that express a limited pattern of EBV antigens, such as Burkitt's-like PTLD and HD-like PTLD tumours which express antigens known to be weakly immunogenic. An important additional step would therefore be to evaluate the expression of EBV antigens present on each tumour using immunohistochemical methods. This could involve use of the novel monoclonal antibody RFD3 shown in this study (**Chapter 6**) to detect the presence of EBV in all forms of latent infection.

Prospective assessment of a larger cohort of PTLD patients is required to fully evaluate ability of the ICS assay to detect functional EBV immunity in all permutations of PTLD encountered in patients. Study of a larger patient cohort may also allow determination of a critical threshold number of IFN- γ producing EBV-specific CD8⁺ T cells required to control PTLD.

Detection of functional EBV-specific T cells by intracellular staining for cytokine production also has wider clinical applications. Experiments described in this thesis have suggested that detection of IFN- γ producing EBV-specific T cells after stimulation with EBV antigen is also possible in TILs and PBMC samples from EBV-positive HD patients. Comparison of results from reactive and EBV-negative HD LNs revealed presence of EBV-specific CD4⁺ T cells and CD8⁺ T cells in the

LN_s expressing EBV antigens, showing that functional EBV-specific T cells are recruited to EBV-positive HD tumours. In order to elucidate the role of these cells, further experiments are required to examine the precise EBV antigen specificity of the T cells and whether they represent a protective immune response with potential to eradicate tumour cells or if infiltrating cells include T cells that suppress anti-tumour immunity, as has been described (Alvaro *et al.*, 2005; Marshall *et al.*, 2004). Detailed assessment of the range of cytokines produced from the EBV-specific T cells found in HD TILs by the flow cytometry based assay or qRT-PCR could be used to address these questions. Further research into the pathogenesis of EBV-driven HD and the precise function of the EBV-specific T cells present in TILs is particularly important for the future role of adoptive transfer of EBV-specific T cells as a treatment for EBV-positive HD (Bollard *et al.*, 2004; Lucas *et al.*, 2004). Clearly if functional regulatory T cells are present, infused T cells entering the tumour could quickly be suppressed.

Another potential clinical application of ICS for detecting functional antigen specific T cells is in the treatment of HHV8-driven KS. This tumour occurs post transplantation and, analogous to EBV-driven PTLN, is thought to be caused by viral reactivation secondary to suppression of the host's natural immunity (Cattani *et al.*, 2001; Allen, 2002). Post transplant KS is treated effectively with RIS with complete clinical responses in the majority of patients (Euvrard *et al.*, 2003). The immune response to HHV8 has been well characterised in both healthy sero-positive individuals (Osman *et al.*, 1999) and primary infection (Wang *et al.*, 2001) and several HLA class I restricted epitopes have been identified (Ribechini *et al.*, 2006; Micheletti *et al.*, 2002; Wilkinson *et al.*, 2002). Stimulation of PBMCs with HHV8 antigens and the detection of IFN- γ producing HHV8-specific CD8⁺ T cells by ICS

could be used to tailor RIS in KS patients. Although EBV and HHV8 are both γ -herpes viruses and share similar patterns of gene expression, HHV8 is unable to immortalise cells *in vitro*, in the absence of EBV (Kliche *et al.*, 1998). Therefore HLA class I restricted HHV8 peptide pools would have to be used as the antigen source, limiting the assay to patients with HLA types for which epitopes are known. Another factor that would also need to be considered is that the efficiency of peptide presentation by APCs from HHV8-positive patients may be compromised because the virus is known to suppress cell surface expression of HLA class I molecules by reducing TAP expression (Brander *et al.*, 2000; Guihot *et al.*, 2006).

In summary the work described in this thesis has shown that a simple intracellular assay to detect IFN- γ producing EBV-specific T cells is able to measure EBV immunity in healthy individuals and patients with EBV-driven malignancies, and that it could be applied clinically to assist with treatment related decisions.

Appendices

Appendix 1.1

Clinical Staging of Hodgkin's and Non-Hodgkin's Lymphomas (Ann Arbor Classification)

<i>Stage</i>	<i>Distribution of Disease</i>
I	Involvement of a single lymph node region (I) or involvement of a single extra lymphatic organ or site (IE)
II	Involvement of 2 or more lymph node regions on the same side of the diaphragm alone (II) or with involvement of limited contiguous extra lymphatic organ or site (IIE)
III	Involvement of lymph node regions on both sides of the diaphragm (III), which may include the spleen (IIIS) and/or limited contiguous extra lymphatic organ or site (IIIE, IIIES)
IV	Multiple or disseminated foci of involvement of one or more extra lymphatic organ or site (IIIE, IIIES)

All stages are further divided on the basis of the absence (A) or presence (B) of the following systemic symptoms: fever ($>38^{\circ}\text{C}$), night sweats, or weight loss of greater than 10% of body weight.

Adapted from (Carbone *et al.*, 1971).

Appendix 1.2

Karnofsky Scale

<i>Scale (%)</i>	<i>Description</i>
100	No complaints; no evidence of disease
90	Able to carry on with normal activity; minor signs or symptoms of disease
80	Some signs or symptoms of disease with effort
70	Cares for self; unable to carry on with normal activity or to do active work
60	Requires occasional assistance but is able to care for most personal needs
50	Requires considerable assistance and frequent medical care
40	Disabled; requires special care and assistance
30	Severely disabled; hospitalisation indicated, although death not imminent
20	Very sick; hospitalisation necessary; requires active supportive treatment
10	Moribund; fatal processes progressing rapidly
0	Dead

Adapted from (Karnofsky and Burchenal, 1949)

Appendix 1.3

Response evaluation criteria in solid tumours: RECIST criteria

<i>Response</i>	
Complete response (CR)	Disappearance of all target lesions; confirmed at 4 weeks.
Partial response (PR)	At least 30% reduction in the longest diameter (LD) of a single tumour or the sum of the LD of multiple lesions, taking as reference the baseline study; confirmed at 4 weeks.
Stable disease (SD)	Neither PR or PD criteria are met.
Progressive disease (PD)	At least 20% increase in the LD of a single lesion or the sum of the LD of multiple lesions, taking as reference the smallest LD or the sum of the LDs recorded since treatment started, or appearance of new lesions.

Adapted from (Therasse *et al.*, 2000).

Appendix 1.4

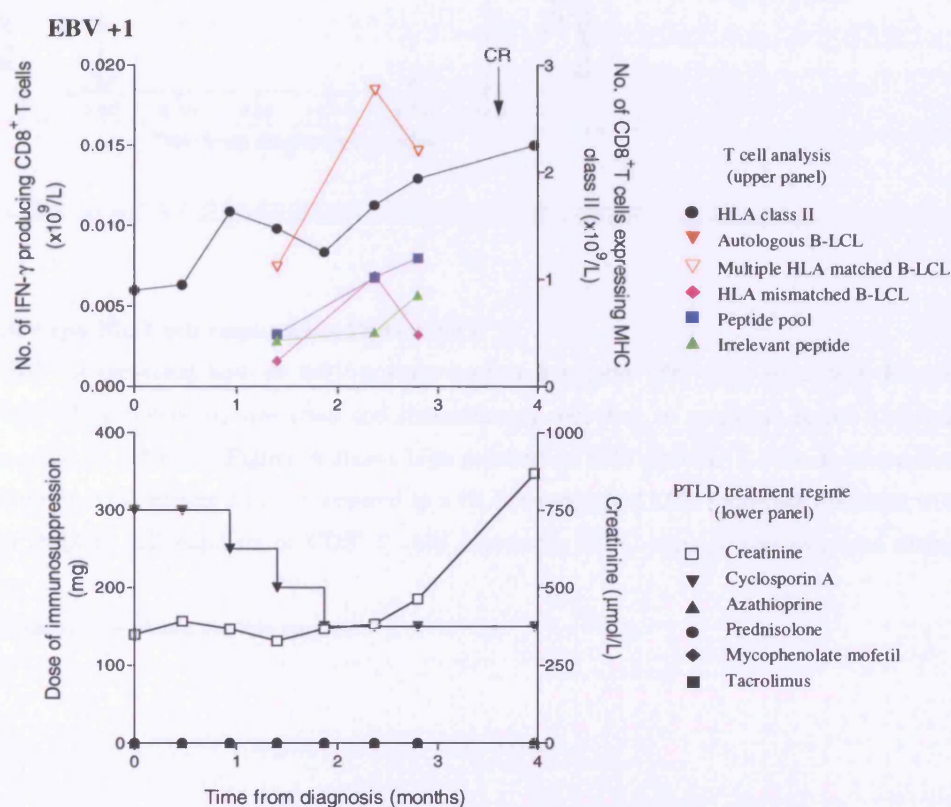
GAPDH Primers

Forward primer: gtc aga cgc atc ttc ttt t

Reverse primer: atc gcc cca ctt gat ttt

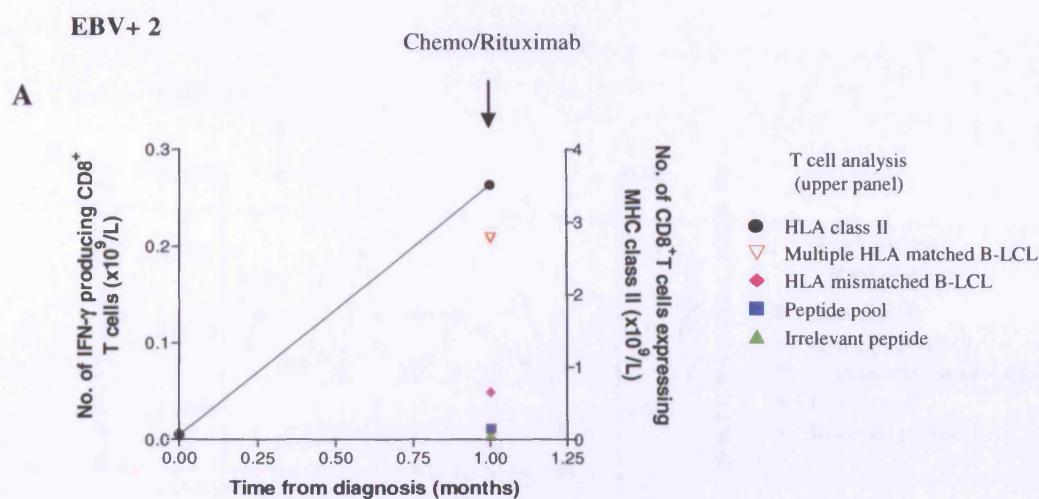
Appendix 1.5

Serial measurement of EBV-specific T cell responses in EBV-positive and EBV-negative PTLD patients



Serial EBV-specific T cell responses in Patient EBV⁺ 1.

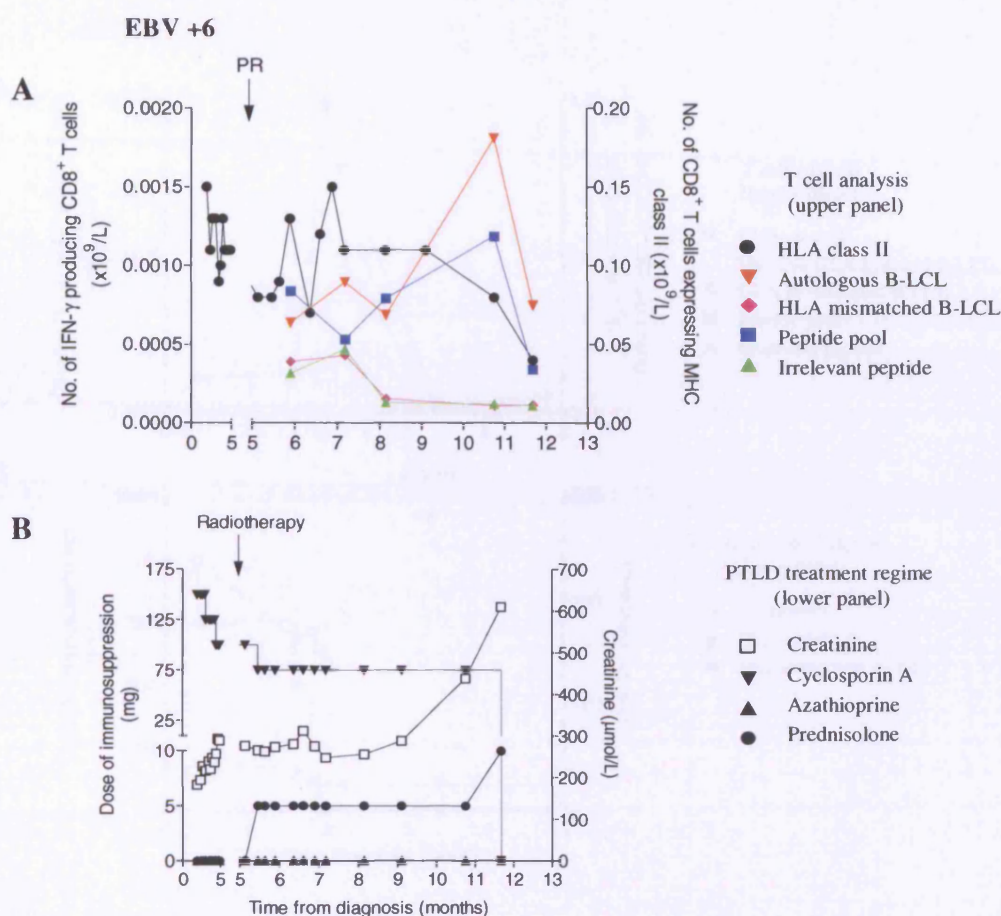
Patient EBV⁺ 1 presented with an EBV-positive tumour 129 months following renal transplant. He was treated with surgery and RIS and had a complete response to treatment. **Figure A** shows a rise in the number of EBV-specific T cells on stimulation with an multiple HLA-matched LCL compared to an HLA-mismatched LCL during treatment. The response to stimulation with an HLA class I restricted EBV peptide pool also increased during treatment compared to an EBV peptide restricted through an irrelevant HLA allele (irrelevant peptide), but this was less than the response seen after stimulation with autologous LCLs. Cell surface expression of the lymphocyte activation marker MHC class II also increased during treatment. **Figure B** shows the RIS schedule and serial creatinine measurements.



Serial EBV-specific T cell responses in Patient EBV⁺ 2

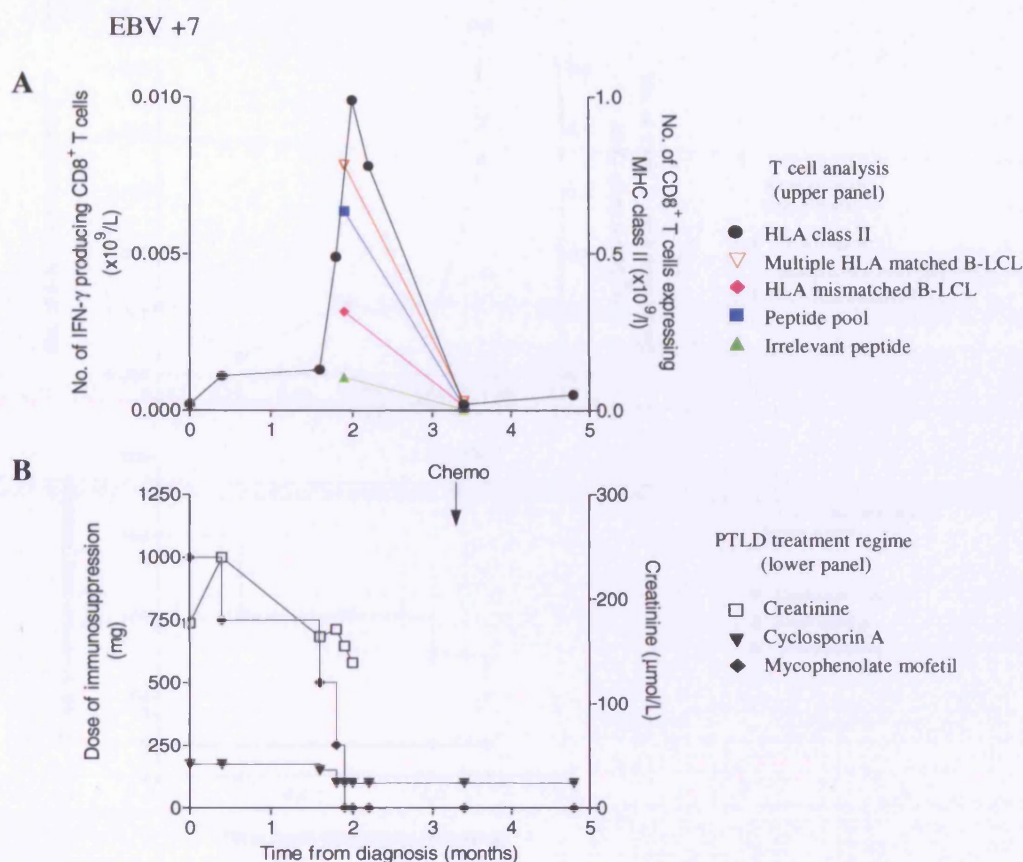
Patient EBV⁺ 2 presented with an EBV-positive tumour 4 months after renal transplant. He was treated with RIS followed by rituximab and chemotherapy, but died on treatment before a clinical response could be achieved. **Figure A** shows high numbers of EBV-specific T cells on stimulation with a multiple HLA-matched LCL compared to a HLA-mismatched LCL following treatment with RIS. The absolute cell numbers of CD8⁺ T cells expressing MHC class II also increased during treatment.

RIS data was not available for this patient.



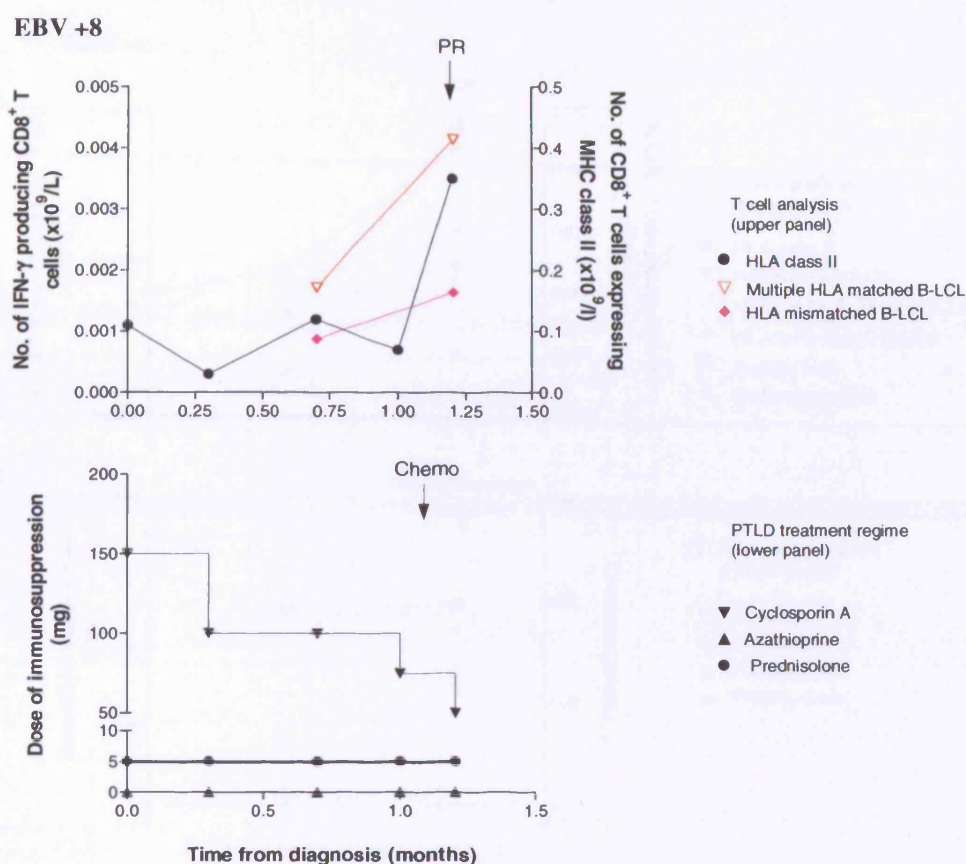
Serial EBV-specific T cell responses in Patient EBV+6.

Patient EBV+6 presented with an EBV-positive tumour 216 months following renal transplant. He was treated with RIS and radiotherapy and had a partial response to treatment. Following this he developed hydronephrosis and renal failure, and died 12 months after PTLD diagnosis. **Figure A** shows a rise in the absolute number of EBV-specific T cells on stimulation with an autologous LCL compared to an HLA-mismatched LCL during treatment with RIS. This trend was mirrored by the response obtained after stimulation with HLA class I restricted EBV peptide pools. The magnitude of the response using peptides however was less than that with autologous LCLs. The number of CD8⁺ T cells expressing the lymphocyte activation marker MHC class II remained the same for the first nine months after diagnosis during treatment with RIS and then fell in conjunction with the number of EBV-specific T cells prior to death. **Figure B** shows the RIS schedule and serial creatinine measurements.



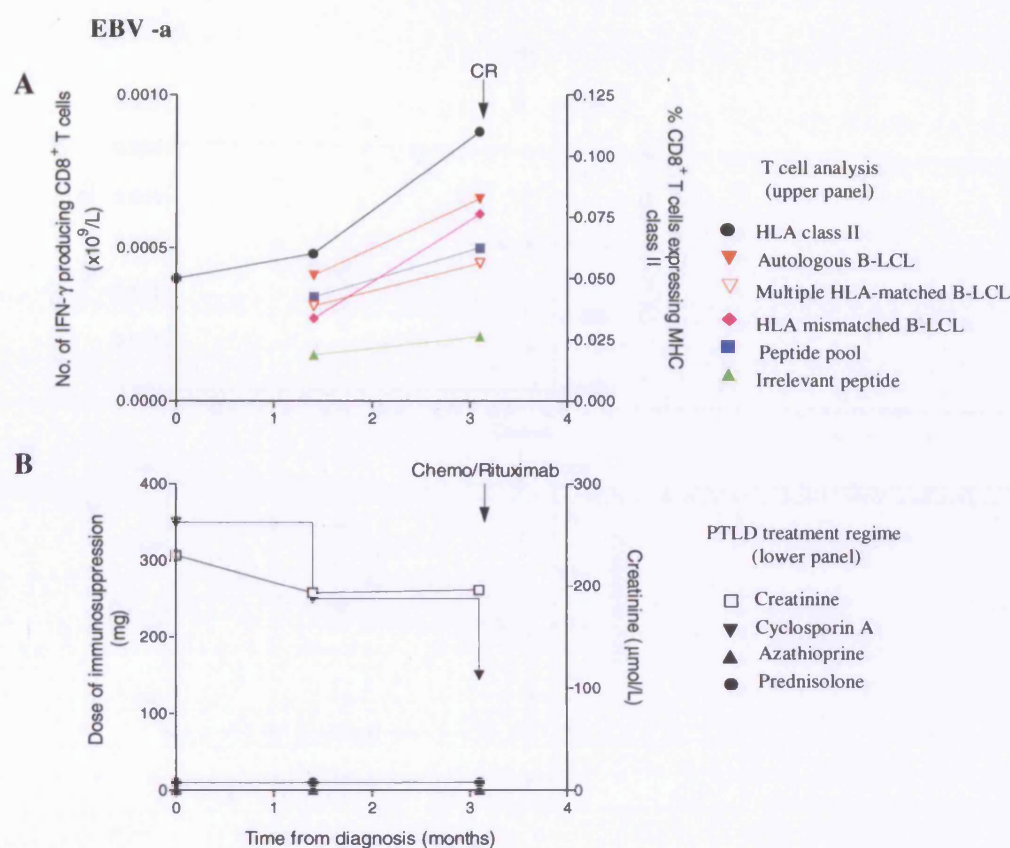
Serial EBV-specific T cell responses in Patient EBV⁺ 7.

Patient EBV⁺ 7 presented with an EBV-positive tumour 39 months following renal transplant. He was treated with RIS followed by cytotoxic chemotherapy and had a partial response to treatment. **Figure A** shows a fall in the absolute number of EBV-specific T cells on stimulation with a 3 allele HLA class I matched LCL and an HLA class I restricted EBV peptide pool during treatment compared to the relevant negative controls. The magnitude of the response using the peptide pool was however less than that with the autologous LCL. The number of CD8⁺ T cells expressing the lymphocyte activation marker MHC class II also fell during treatment. **Figure B** shows the RIS schedule and serial creatinine measurements.



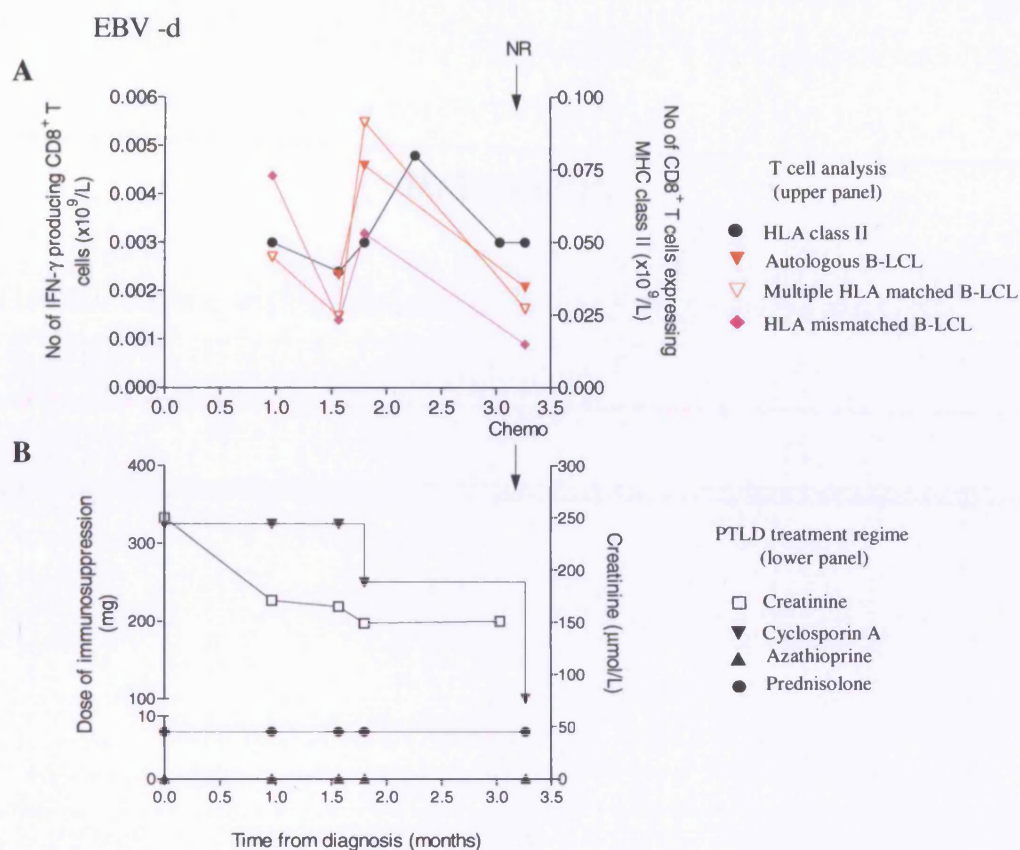
Serial EBV-specific T cell responses in Patient EBV + 8.

Patient EBV + 8 presented with a PTLD tumour 58 months following renal transplant. He was treated with RIS followed by cytotoxic chemotherapy and had a partial response treatment but died due to neutropenic sepsis. **Figure A** shows an increase in the absolute number of EBV-specific T cells on stimulation with a multiple HLA-matched LCL compared to an HLA-mismatched LCL during treatment with RIS. The number of CD8⁺ T cells expressing the lymphocyte activation marker MHC class II also increased during treatment. **Figure B** shows the RIS schedule. Serial creatinine measurements were unavailable.



Serial EBV-specific T cell responses in Patient EBV⁻ a.

Patient EBV⁻ a presented with an EBV-negative tumour 150 months following renal transplant. She was treated with RIS and rituximab and had a complete response to treatment. **Figure A** shows no rise in the number of EBV-specific T cells on stimulation with an autologous LCL or a multiple HLA-matched LCL compared to an HLA-mismatched LCL during treatment with RIS. The number of CD8⁺ T cells expressing the lymphocyte activation marker MHC class II was also unchanged. **Figure B** shows the RIS schedule and serial creatinine measurements.



Serial EBV-specific T cell responses in Patient EBV⁻d

Patient EBV⁻d presented with an EBV-negative tumour 143 months following renal transplant. He was initially treated with RIS but had no clinical response. He was then treated with 6 cycles of chemotherapy and had a complete clinical response. **Figure A** shows no consistent rise in the number of EBV-specific T cells on stimulation with an autologous LCL and a 3 allele HLA class I matched LCL compared to an HLA-mismatched LCL during treatment with RIS. There was also no consistent rise in the number of CD8⁺ T cells expressing the lymphocyte activation marker MHC class II. **Figure B** shows the RIS schedule and serial creatinine measurements.

References

Reference List

- Abdalla,A.O., Kiaii,S., Hansson,L., Rossmann,E.D., Jeddi-Tehrani,M., Shokri,F., Osterborg,A., Mellstedt,H., and Rabbani,H. (2003). Kinetics of cytokine gene expression in human CD4+ and CD8+ T-lymphocyte subsets using quantitative real-time PCR. *Scand. J Immunol* 58, 601-606.
- Alber,G., Kim,K.M., Weiser,P., Riesterer,C., Carsetti,R., and Reth,M. (1993). Molecular mimicry of the antigen receptor signalling motif by transmembrane proteins of the Epstein-Barr virus and the bovine leukaemia virus. *Curr. Biol.* 3, 333-339.
- Aleman,B.M.P., Raemaekers,J.M.M., Tirelli,U., Bortolus,R., 't Veer,M.B., Lybeert,M.L.M., Keuning,J.J., Carde,P., Girinsky,T., van der Maazen,R.W.M., Tomsic,R., Vovk,M., van Hoof,A., Demeestere,G., Lugtenburg,P.J., Thomas,J., Schroyens,W., De Boeck,K., Baars,J.W., Kluin-Nelemans,J.C., Carrie,C., Aoudjhane,M., Bron,D., Eghbali,H., Smit,W.G.J.M., Meerwaldt,J.H., Hagenbeek,A., Pinna,A., Henry-Amar,M., and the European Organization for Research and Treatment of Cancer Lymphoma Group (2003). Involved-Field Radiotherapy for Advanced Hodgkin's Lymphoma. *The New England Journal Of Medicine* 348, 2396-2406.
- Alfieri,C., Tanner,J., Carpentier,L., Perpete,C., Savoie,A., Paradis,K., Delage,G., and Joncas,J. (1996). Epstein-Barr virus transmission from a blood donor to an organ transplant recipient with recovery of the same virus strain from the recipient's blood and oropharynx. *Blood* 87, 812-817.
- Allen,U., Hebert,D., Petric,M., Tellier,R., Tran,D., Superina,R., Stephens,D., West,L., Wasfy,S., and Nelson,S. (2001). Utility of semiquantitative polymerase chain reaction for Epstein-Barr virus to measure virus load in pediatric organ transplant recipients with and without posttransplant lymphoproliferative disease. *Clin. Infect. Dis.* 33, 145-150.
- Allen,U.D. (2002). Human herpesvirus type 8 infections among solid organ transplant recipients. *Pediatric Transplantation* 6, 187-192.
- Altman,J.D., Moss,P.A., Goulder,P.J., Barouch,D.H., McHeyzer-Williams,M.G., Bell,J.I., McMichael,A.J., and Davis,M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94-96.
- Alvarez,I., Sureda,A., Caballero,M.D., Urbano-Ispizua,A., Ribera,J.M., Canales,M., Garcia-Conde,J., Sanz,G., Arranz,R., and Bernal,M.T. (2006). Nonmyeloablative Stem Cell Transplantation Is an Effective Therapy for Refractory or Relapsed Hodgkin Lymphoma: Results of a Spanish Prospective Cooperative Protocol. *Biology of Blood and Marrow Transplantation* 12, 172-183.
- Alvaro,T., Lejeune,M., Salvado,M.T., Bosch,R., Garcia,J.F., Jaen,J., Banham,A.H., Roncador,G., Montalban,C., and Piris,M.A. (2005). Outcome in Hodgkin's

References

Lymphoma Can Be Predicted from the Presence of Accompanying Cytotoxic and Regulatory T Cells. *Clin Cancer Res* 11, 1467-1473.

Ambinder,R.F. (2000). Gammaherpesviruses and "Hit-and-Run" oncogenesis. *Am. J. Pathol.* 156, 1-3.

Amlot,P.L. (2001). Epstein-Barr virus and post-transplant lymphomas. *Cancer Topics* 11, 12.

Amlot,P.L., Tahami,F., Chinn,D., and Rawlings,E. (1996). Activation antigen expression on human T cells. I. Analysis by two-colour flow cytometry of umbilical cord blood, adult blood and lymphoid tissue. *Clin. Exp. Immunol.* 105, 176-182.

Amlot, P. L., Tahami, F., Rawlings, E., Rees, L., Venkat-Raman, G., and Sweny, P. Optimising reduction of immunosuppression in the management of posttransplant lymphoproliferative disease (PTLD) in renal ransplant patients. *N Engl J Med* . 2007.

Ref Type: In Press

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., and Callan,M.F. (2003). Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection. *J. Exp. Med.* 198, 903-911.

Amyes,E., McMichael,A.J., and Callan,M.F.C. (2005). Human CD4+ T Cells Are Predominantly Distributed among Six Phenotypically and Functionally Distinct Subsets. *J Immunol* 175, 5765-5773.

Anderlini,P., Acholonu,S.A., Okorogi,G.J., Andersson,B.S., Couriel,D.R., De Lima,M.J., Donato,M.L., Khouri,I.F., Giral,S.A., Ueno,N.T., and Champlin,R.E. (2004). Donor leukocyte infusions in relapsed Hodgkin's lymphoma following allogeneic stem cell transplantation: CD3+ cell dose, GVHD and disease response. *Bone Marrow Transplant.* 34, 511-514.

Anderlini,P., Saliba,R., Acholonu,S., Okorogi,G.J., Donato,M., Giral,S., Andersson,B., Ueno,N.T., Khouri,I., and Lima et,a. (2005). Reduced-intensity allogeneic stem cell transplantation in relapsed and refractory Hodgkin's disease: low transplant-related mortality and impact of intensity of conditioning regimen. *Bone Marrow Transplantation* 35, 943-951.

Anichini,A., Molla,A., Mortarini,R., Tragni,G., Bersani,I., Di Nicola,M., Gianni,A.M., Pilotti,S., Dunbar,R., Cerundolo,V., and Parmiani,G. (1999). An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. *J Exp. Med.* 190, 651-667.

Annels,N.E., Callan,M.F.C., Tan,L., and Rickinson,A.B. (2000). Changing Patterns of Dominant TCR Usage with Maturation of an EBV-Specific Cytotoxic T Cell Response. *J Immunol* 165, 4831-4841.

References

- Araujo,I., Foss,H.D., Hummel,M., Anagnostopoulos,I., Barbosa,H.S., Bittencourt,A., and Stein,H. (1999). Frequent expansion of Epstein-Barr virus (EBV) infected cells in germinal centres of tonsils from an area with a high incidence of EBV-associated lymphoma. *J Pathol* 187, 326-330.
- Armstrong,R.W., Imrey,P.B., Lye,M.S., Armstrong,M.J., Yu,M.C., and Sani,S. (1998). Nasopharyngeal carcinoma in Malaysian Chinese: salted fish and other dietary exposures. *Int. J Cancer* 77, 228-235.
- Babcock,G.J., Decker,L.L., Freeman,R.B., and Thorley-Lawson,D.A. (1999). Epstein-barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. *J Exp. Med.* 190, 567-576.
- Babcock,G.J., Decker,L.L., Volk,M., and Thorley-Lawson,D.A. (1998). EBV persistence in memory B cells in vivo. *Immunity.* 9, 395-404.
- Babcock,G.J., Hochberg,D., and Thorley-Lawson,A.D. (2000). The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. *Immunity.* 13, 497-506.
- Babcock,G.J. and Thorley-Lawson,D.A. (2000). Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors. *Proc. Natl Acad. Sci. U. S. A* 97, 12250-12255.
- Baer,R., Bankier,A.T., Biggin,M.D., Deininger,P.L., Farrell,P.J., Gibson,T.J., Hatfull,G., Hudson,G.S., Satchwell,S.C., Seguin,C., and . (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* 310, 207-211.
- Baudouin,V., Dehee,A., Pedron-Grossetete,B., Ansart-Pirenne,H., Haddad,E., Maisin,A., Loirat,C., and Sterkers,G. (2004). Relationship between CD8+ T-cell phenotype and function, Epstein-Barr virus load, and clinical outcome in pediatric renal transplant recipients: a prospective study. *Transplantation.* 77, 1706-1713.
- Bejarano,M.T. and Masucci,M.G. (1998). Interleukin-10 Abrogates the Inhibition of Epstein-Barr Virus-Induced B-Cell Transformation by Memory T-Cell Responses. *Blood* 92, 4256-4262.
- Benden,C., Aurora,P., Burch,M., Cubitt,D., Lloyd,C., Whitmore,P., Neligan,S.L., and Elliott,M.J. (2005). Monitoring of Epstein-Barr viral load in pediatric heart and lung transplant recipients by real-time polymerase chain reaction. *J Heart Lung Transplant.* 24, 2103-2108.
- Benkerrou,M., Jais,J.P., Leblond,V., Durandy,A., Sutton,L., Bordigoni,P., Garnier,J.L., Le Bidois,J., Le Deist,F., Blanche,S., and Fischer,A. (1998). Anti-B-Cell Monoclonal Antibody Treatment of Severe Posttransplant B-Lymphoproliferative Disorder: Prognostic Factors and Long-Term Outcome. *Blood* 92, 3137-3147.
- Betts,M.R., Ambrozak,D.R., Douek,D.C., Bonhoeffer,S., Brenchley,J.M., Casazza,J.P., Koup,R.A., and Picker,L.J. (2001). Analysis of total human

References

- immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J. Virol.* 75, 11983-11991.
- Beynet,D.P., Wee,S.A., Horwitz,S.S., Kohler,S., Horning,S., Hoppe,R., and Kim,Y.H. (2004). Clinical and pathological features of posttransplantation lymphoproliferative disorders presenting with skin involvement in 4 patients. *Arch. Dermatol.* 140, 1140-1146.
- Bhatia,K.G., Gutierrez,M.I., Huppi,K., Siwarski,D., and Magrath,I.T. (1992). The pattern of p53 mutations in Burkitt's lymphoma differs from that of solid tumors. *Cancer Res* 52, 4273-4276.
- Bhatia,S., Ramsay,N.K., Steinbuch,M., Dusenbery,K.E., Shapiro,R.S., Weisdorf,D.J., Robison,L.L., Miller,J.S., and Neglia,J.P. (1996). Malignant neoplasms following bone marrow transplantation. *Blood* 87, 3633-3639.
- Biggar,R.J., Henle,G., Bocker,J., Lennette,E.T., Fleisher,G., and Henle,W. (1978). Primary Epstein-Barr virus infections in African infants. II. Clinical and serological observations during seroconversion. *Int. J Cancer* 22, 244-250.
- Blake,N., Lee,S., Redchenko,I., Thomas,W., Steven,N., Leese,A., Steigerwald-Mullen,P., Kurilla,M.G., Frappier,L., and Rickinson,A. (1997). Human CD8+ T cell responses to EBV EBNA1: HLA class I presentation of the (Gly-Ala)-containing protein requires exogenous processing. *Immunity.* 7, 791-802.
- Blomberg,J., Andersson,M., and Faldt,R. (1987). Differential pattern of oncogene and beta-actin expression in leukaemic cells from AML patients. *Br J Haematol.* 65, 83-86.
- Bofill,M., Akbar,A.N., and Amlot,P.L. (2000). Follicular dendritic cells share a membrane-bound protein with fibroblasts. *J Pathol* 191, 217-226.
- Boll,B., Hansen,H., Heuck,F., Reiners,K., Borchmann,P., Rothe,A., Engert,A., and Pogge von Strandmann,E. (2005). The fully human anti-CD30 antibody 5F11 activates NF- κ B and sensitizes lymphoma cells to bortezomib-induced apoptosis. *Blood* 106, 1839-1842.
- Bollard,C.M., Aguilar,L., Straathof,K.C., Gahn,B., Huls,M.H., Rousseau,A., Sixbey,J., Gresik,M.V., Carrum,G., Hudson,M., Dilloo,D., Gee,A., Brenner,M.K., Rooney,C.M., and Heslop,H.E. (2004). Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J Exp. Med.* 200, 1623-1633.
- Bollard,C.M., Gottschalk,S., Huls,M.H., Molldrem,J., Przepiorka,D., Rooney,C.M., and Heslop,H.E. (2006). In vivo expansion of LMP 1- and 2-specific T-cells in a patient who received donor-derived EBV-specific T-cells after allogeneic stem cell transplantation. *Leuk. Lymphoma* 47, 837-842.
- Borchmann,P., Schnell,R., Fuss,I., Manzke,O., Davis,T., Lewis,L.D., Behnke,D., Wickenhauser,C., Schiller,P., Diehl,V., and Engert,A. (2002). Phase 1 trial of the novel bispecific molecule H22xKi-4 in patients with refractory Hodgkin lymphoma. *Blood* 100, 3101-3107.

References

- Borchmann,P., Trembl,J.F., Hansen,H., Gottstein,C., Schnell,R., Staak,O., Zhang,H.f., Davis,T., Keler,T., Diehl,V., Graziano,R.F., and Engert,A. (2003). The human anti-CD30 antibody 5F11 shows in vitro and in vivo activity against malignant lymphoma. *Blood* 102, 3737-3742.
- Brander,C., Suscovich,T., Lee,Y., Nguyen,P.T., O'Connor,P., Seebach,J., Jones,N.G., van Gorder,M., Walker,B.D., and Scadden,D.T. (2000). Impaired CTL Recognition of Cells Latently Infected with Kaposi's Sarcoma-Associated Herpes Virus. *J Immunol* 165, 2077-2083.
- Brauninger,A., Spieker,T., Mottok,A., Baur,A.S., Kuppers,R., and Hansmann,M.L. (2003). Epstein-Barr virus (EBV)-positive lymphoproliferations in post-transplant patients show immunoglobulin V gene mutation patterns suggesting interference of EBV with normal B cell differentiation processes. *Eur. J Immunol* 33, 1593-1602.
- Brooks,L.A., Lear,A.L., Young,L.S., and Rickinson,A.B. (1993). Transcripts from the Epstein-Barr virus BamHI A fragment are detectable in all three forms of virus latency. *J Virol.* 67, 3182-3190.
- Brown,M.H., Boles,K., Anton van der Merwe,P., Kumar,V., Mathew,P.A., and Neil Barclay,A. (1998). 2B4, the Natural Killer and T Cell Immunoglobulin Superfamily Surface Protein, Is a Ligand for CD48. *J. Exp. Med.* 188, 2083-2090.
- Brunner,K.T., Mael,J., Cerottini,J.C., and Chapuis,B. (1968). Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* 14, 181-196.
- Budiani,D.R., Hutahaeen,S., Haryana,S.M., Soesatyo,M.H., and Sosroseno,W. (2002). Interleukin-10 levels in Epstein-Barr virus-associated nasopharyngeal carcinoma. *J Microbiol. Immunol Infect.* 35, 265-268.
- Buell,J.F., Gross,T.G., Hanaway,M.J., Trofe,J., Roy-Chaudhury,P., First,M.R., and Woodle,E.S. (2005). Posttransplant lymphoproliferative disorder: significance of central nervous system involvement. *Transplant. Proc.* 37, 954-955.
- BURKITT,D. (1958). A sarcoma involving the jaws in African children. *Br J Surg.* 46, 218-223.
- Bustin,S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25, 169-193.
- Caillard,S., Lachat,V., and Moulin,B. (2000). Posttransplant lymphoproliferative disorders in renal allograft recipients: report of 53 cases of a French multicenter study. PTLTD French Working Group. *Transpl. Int.* 13 Suppl 1, S388-S393.
- Callan,M.F., Annels,N., Steven,N., Tan,L., Wilson,J., McMichael,A.J., and Rickinson,A.B. (1998a). T cell selection during the evolution of CD8+ T cell memory in vivo. *Eur. J Immunol* 28, 4382-4390.
- Callan,M.F., Fazou,C., Yang,H., Rostron,T., Poon,K., Hatton,C., and McMichael,A.J. (2000). CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *J Clin. Invest* 106, 1251-1261.

References

- Callan,M.F., Steven,N., Krausa,P., Wilson,J.D., Moss,P.A., Gillespie,G.M., Bell,J.I., Rickinson,A.B., and McMichael,A.J. (1996). Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. *Nature Medicine* 2, 906-911.
- Callan,M.F.C., Tan,L., Annels,N., Ogg,G.S., Wilson,J.D.K., O'Callaghan,C.A., Steven,N., McMichael,A.J., and Rickinson,A.B. (1998b). Direct Visualization of Antigen-specific CD8+ T Cells during the Primary Immune Response to Epstein-Barr Virus In Vivo. *J. Exp. Med.* 187, 1395-1402.
- Canellos,G.P., Anderson,J.R., Propert,K.J., Nissen,N., Cooper,M.R., Henderson,E.S., Green,M.R., Gottlieb,A., and Peterson,B.A. (1992). Chemotherapy of advanced Hodgkin's disease with MOPP, ABVD, or MOPP alternating with ABVD. *N Engl J Med* 327, 1478-1484.
- Canellos,G.P. and Niedzwiecki,D. (2002). Long-term follow-up of Hodgkin's disease trial. *N Engl J Med* 346, 1417-1418.
- Capello,D., Rossi,D., and Gaidano,G. (2005). Post-transplant lymphoproliferative disorders: molecular basis of disease histogenesis and pathogenesis. *Hematol. Oncol* 23, 61-67.
- Carbone,P.P., Kaplan,H.S., Musshoff,K., Smithers,D.W., and Tubiana,M. (1971). Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res* 31, 1860-1861.
- Carpentier,L., Tapiero,B., Alvarez,F., Viau,C., and Alfieri,C. (2003). Epstein-Barr virus (EBV) early-antigen serologic testing in conjunction with peripheral blood EBV DNA load as a marker for risk of posttransplantation lymphoproliferative disease. *J. Infect. Dis.* 188, 1853-1864.
- Catalina,M.D., Sullivan,J.L., Bak,K.R., and Luzuriaga,K. (2001). Differential Evolution and Stability of Epitope-Specific CD8+ T Cell Responses in EBV Infection. *J Immunol* 167, 4450-4457.
- Cattani,P., Capuano,M., Graffeo,R., Ricci,R., Cerimele,F., Cerimele,D., Nanni,G., and Fadda,G. (2001). Kaposi's Sarcoma Associated with Previous Human Herpesvirus 8 Infection in Kidney Transplant Recipients. *J. Clin. Microbiol.* 39, 506-508.
- Cavazzana,C., Bensoussan, Jabado, Haddad, Yvon, Moskwa, Tachet,D.C., Buisson, Morand, Virion, Deist,L.E., and Fischer (1998). Prevention of EBV-induced B-lymphoproliferative disorder by ex vivo marrow B-cell depletion in HLA-phenotypical or non-identical T-depleted bone marrow transplantation. *British Journal of Haematology* 103, 543-551.
- Cen,H., Breinig,M.C., Atchison,R.W., Ho,M., and McKnight,J.L. (1991). Epstein-Barr virus transmission via the donor organs in solid organ transplantation: polymerase chain reaction and restriction fragment length polymorphism analysis of IR2, IR3, and IR4. *Journal Of Virology* 65, 976-980.
- Cen,H., Williams,P.A., McWilliams,H.P., Breinig,M.C., Ho,M., and McKnight,J.L. (1993). Evidence for restricted Epstein-Barr virus latent gene expression and anti-

References

EBNA antibody response in solid organ transplant recipients with posttransplant lymphoproliferative disorders. *Blood* 81, 1393-1403.

Cesarman,E., Nador,R.G., Bai,F., Bohenzky,R.A., Russo,J.J., Moore,P.S., Chang,Y., and Knowles,D.M. (1996). Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. *J. Virol.* 70, 8218-8223.

Cesarman,E., Chadburn,A., Liu,Y.F., Migliazza,A., Dalla-Favera,R., and Knowles,D.M. (1998). BCL-6 Gene Mutations in Posttransplantation Lymphoproliferative Disorders Predict Response to Therapy and Clinical Outcome. *Blood* 92, 2294-2302.

Chadburn,A., Chen,J.M., Hsu,D.T., Frizzera,G., Cesarman,E., Garrett,T.J., Mears,J.G., Zangwill,S.D., Addonizio,L.J., Michler,R.E., and Knowles,D.M. (1998). The morphologic and molecular genetic categories of posttransplantation lymphoproliferative disorders are clinically relevant. *Cancer* 82, 1978-1987.

Chan,A.T.C., Teo,P.M.L., and Johnson,P.J. (2002). Nasopharyngeal carcinoma. *Annals of Oncology* 13, 1007-1015.

Chapman,A.L., Rickinson,A.B., Thomas,W.A., Jarrett,R.F., Crocker,J., and Lee,S.P. (2001). Epstein-Barr virus-specific cytotoxic T lymphocyte responses in the blood and tumor site of Hodgkin's disease patients: implications for a T-cell-based therapy. *Cancer Res.* 61, 6219-6226.

Chatila,T., Wong,R., Young,M., Miller,R., Terhorst,C., and Geha,R.S. (1989). An immunodeficiency characterized by defective signal transduction in T lymphocytes. *The New England Journal Of Medicine* 320, 696-702.

Chen,H., Lee,J.M., Wang,Y., Huang,D.P., Ambinder,R.F., and Hayward,S.D. (1999). The Epstein-Barr virus latency BamHI-Q promoter is positively regulated by STATs and Zta interference with JAK/STAT activation leads to loss of BamHI-Q promoter activity. *Proc. Natl. Acad. Sci. U. S. A* 96, 9339-9344.

Choquet,S., Leblond,V., Herbrecht,R., Socie,G., Stoppa,A.M., Vandenberghe,P., Fischer,A., Morschhauser,F., Salles,G., Feremans,W., Vilmer,E., Peraldi,M.N., Lang,P., Lebranchu,Y., Oksenhendler,E., Garnier,J.L., Lamy,T., Jaccard,A., Ferrant,A., Offner,F., Hermine,O., Moreau,A., Fafi-Kremer,S., Morand,P., Chatenoud,L., Berriot-Varoqueaux,N., Bergounoux,L., and Milpied,N. (2006). Efficacy and safety of rituximab in B-cell post-transplantation lymphoproliferative disorders: results of a prospective multicenter phase 2 study. *Blood* 107, 3053-3057.

Choquet,S., Mamzer,B.M., Hermine,O., Porcher,R., Nguyen,Q.S., Davi,F., Charlotte,F., Dorent,R., Barrou,B., Vernant,J.P., Raphael,M., Levy,V., and Leblond,V. (2002). Identification of prognostic factors in post-transplant lymphoproliferative disorders. *Recent Results Cancer Res* 159, 67-80.

Cinti,C., Leoncini,L., Nyongo,A., Ferrari,F., Lazzi,S., Bellan,C., Vatti,R., Zamparelli,A., Cevenini,G., osi,G.M., Claudio,P.P., Maraldi,N.M., Tosi,P., and Giordano,A. (2000). Genetic Alterations of the Retinoblastoma-Related Gene

References

RB2/p130 Identify Different Pathogenetic Mechanisms in and among Burkitt's Lymphoma Subtypes. *Am J Pathol* 156, 751-760.

Clave, E., Agbalika, F., Bajzik, V., de Latour, R.P., Trillard, M., Rabian, C., Scieux, C., Devergie, A., Socie, G., Ribaud, P., Ades, L., Ferry, C., Gluckman, E., Charron, D., Esperou, H., Toubert, A., and Moins-Teisserenc, H. (2004). Epstein-Barr virus (EBV) reactivation in allogeneic stem-cell transplantation: relationship between viral load, EBV-specific T-cell reconstitution and rituximab therapy. *Transplantation* 77, 76-84.

Coffey, A.J., Brooksbank, R.A., Brandau, O., Ohashi, T., Howell, G.R., Bye, J.M., Cahn, A.P., Durham, J., Heath, P., Wray, P., Pavitt, R., Wilkinson, J., Leversha, M., Huckle, E., Shaw-Smith, C.J., Dunham, A., Rhodes, S., Schuster, V., Porta, G., Yin, L., Serafini, P., Sylla, B., Zollo, M., Franco, B., Bolino, A., Seri, M., Lanyi, A., Davis, J.R., Webster, D., Harris, A., Lenoir, G., St Basile, G., Jones, A., Behlorsky, B.H., Achatz, H., Murken, J., Fassler, R., Sumegi, J., Romeo, G., Vaudin, M., Ross, M.T., Meindl, A., and Bentley, D.R. (1998). Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet* 20, 129-135.

Cohen, J.I. and Lekstrom, K. (1999). Epstein-Barr Virus BART1 Protein Is Dispensable for B-Cell Transformation and Inhibits Alpha Interferon Secretion from Mononuclear Cells. *J. Virol.* 73, 7627-7632.

Cohen, J.I., Wang, F., Mannick, J., and Kieff, E. (1989). Epstein-Barr Virus Nuclear Protein 2 is a Key Determinant of Lymphocyte Transformation. *PNAS* 86, 9558-9562.

Coiffier, B., Lepage, E., Briere, J., Herbrecht, R., Tilly, H., Bouabdallah, R., Morel, P., Van Den Neste, E., Salles, G., Gaulard, P., Reyes, F., Lederlin, P., and Gisselbrecht, C. (2002). CHOP Chemotherapy plus Rituximab Compared with CHOP Alone in Elderly Patients with Diffuse Large-B-Cell Lymphoma. *The New England Journal Of Medicine* 346, 235-242.

Comoli, P., Pedrazzoli, P., Maccario, R., Basso, S., Carminati, O., Labirio, M., Schiavo, R., Secondino, S., Frasson, C., Perotti, C., Moroni, M., Locatelli, F., and Siena, S. (2005). Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes. *J Clin. Oncol.* 23, 8942-8949.

Comoli, P., Labirio, M., Basso, S., Baldanti, F., Grossi, P., Furione, M., Vigano, M., Fiocchi, R., Rossi, G., Ginevri, F., Gridelli, B., Moretta, A., Montagna, D., Locatelli, F., Gerna, G., and Maccario, R. (2002). Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. *Blood* 99, 2592-2598.

Cook, R.C., Connors, J.M., Gascoyne, R.D., Fradet, G., and Levy, R.D. (1999). Treatment of post-transplant lymphoproliferative disease with rituximab monoclonal antibody after lung transplantation. *Lancet* 354, 1698-1699.

References

- Cote,T.R., Manns,A., Hardy,C.R., Yellin,F.J., Hartge,P., and AIDS/Cancer Study Group (1996). Epidemiology of Brain Lymphoma Among People With or Without Acquired Immunodeficiency Syndrome. *J. Natl. Cancer Inst.* 88, 675-679.
- Cox,K.L., Lawrence-Miyasaki,L.S., Garcia-Kennedy,R., Lennette,E.T., Martinez,O.M., Krams,S.M., Berquist,W.E., So,S.K., and Esquivel,C.O. (1995). An increased incidence of Epstein-Barr virus infection and lymphoproliferative disorder in young children on FK506 after liver transplantation. *Transplantation* 59, 524-529.
- Crawford,D.H. and Ando,I. (1986). EB virus induction is associated with B-cell maturation. *Immunology* 59, 405-409.
- Crawford,D.H., Brickell,P., Tidman,N., McConnell,I., Hoffbrand,A.V., and Janossy,G. (1981). Increased numbers of cells with suppressor T cell phenotype in the peripheral blood of patients with infectious mononucleosis. *Clin Exp. Immunol* 43, 291-297.
- Crawford,D.H., Thomas,J.A., Janossy,G., Sweny,P., Fernando,O.N., Moorhead,J.F., and Thompson,J.H. (1980). Epstein Barr virus nuclear antigen positive lymphoma after cyclosporin A treatment in patient with renal allograft. *Lancet* 1, 1355-1356.
- Crawford,D.H., Macsween,K.F., Higgins,C.D., Thomas,R., McAulay,K., Williams,H., Harrison,N., Reid,S., Conacher,M., Douglas,J., and Swerdlow,A.J. (2006). A Cohort Study among University Students: Identification of Risk Factors for Epstein-Barr Virus Seroconversion and Infectious Mononucleosis. *Clin Infect Dis* 43, 276-282.
- Crawford,D.H., Swerdlow,A.J., Higgins,C., McAulay,K., Harrison,N., Williams,H., Britton,K., and Macsween,K.F. (2002). Sexual history and Epstein-Barr virus infection. *The Journal Of Infectious Diseases* 186, 731-736.
- Crotzer,V.L., Christian,R.E., Brooks,J.M., Shabanowitz,J., Settlege,R.E., Marto,J.A., White,F.M., Rickinson,A.B., Hunt,D.F., and Engelhard,V.H. (2000). Immunodominance among EBV-derived epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. *J Immunol* 164, 6120-6129.
- Curry,J., McHale,C., and Smith,M.T. (2002). Low efficiency of the Moloney murine leukemia virus reverse transcriptase during reverse transcription of rare t(8;21) fusion gene transcripts. *Biotechniques* 32, 768, 770, 772, 754-768, 770, 772, 755.
- Curtis,R.E., Travis,L.B., Rowlings,P.A., Socie,G., Kingma,D.W., Banks,P.M., Jaffe,E.S., Sale,G.E., Horowitz,M.M., Witherspoon,R.P., Shriner,D.A., Weisdorf,D.J., Kolb,H.J., Sullivan,K.M., Sobocinski,K.A., Gale,R.P., Hoover,R.N., Fraumeni,J.F., Jr., and Deeg,H.J. (1999). Risk of Lymphoproliferative Disorders After Bone Marrow Transplantation: A Multi-Institutional Study. *Blood* 94, 2208-2216.
- Darenkov,I.A., Marcarelli,M.A., Basadonna,G.P., Friedman,A.L., Lorber,K.M., Howe,J.G., Crouch,J., Bia,M.J., Klinger,A.S., and Lorber,M.I. (1997). Reduced

References

incidence of Epstein-Barr virus-associated posttransplant lymphoproliferative disorder using preemptive antiviral therapy. *Transplantation* 64, 848-852.

Davenport,M.P., Fazou,C., McMichael,A.J., and Callan,M.F.C. (2002). Clonal Selection, Clonal Senescence, and Clonal Succession: The Evolution of the T Cell Response to Infection with a Persistent Virus. *J Immunol* 168, 3309-3317.

Davis,C.L., Harrison,K.L., McVicar,J.P., Forg,P.J., Bronner,M.P., and Marsh,C.L. (1995). Antiviral prophylaxis and the Epstein Barr virus-related post-transplant lymphoproliferative disorder. *Clin Transplant.* 9, 53-59.

Davis,C.L., Wood,B.L., Sabath,D.E., Joseph,J.S., Stehman-Breen,C., and Broudy,V.C. (1998). Interferon-alpha treatment of posttransplant lymphoproliferative disorder in recipients of solid organ transplants. *Transplantation* 66, 1770-1779.

Davis,J.E., Sutton,V.R., Smyth,M.J., and Trapani,J.A. (2000). Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1. *Cell Death. Differ.* 7, 973-983.

Davis,T.A., Czerwinski,D.K., and Levy,R. (1999). Therapy of B-Cell Lymphoma with Anti-CD20 Antibodies Can Result in the Loss of CD20 Antigen Expression. *Clin Cancer Res* 5, 611-615.

Denzin,L.K. and Cresswell,P. (1995). HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell* 82, 155-165.

Devergne,O., Birkenbach,M., and Kieff,E. (1997). Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *PNAS* 94, 12041-12046.

Devergne,O., Coulomb-L'Hermine,A., Capel,F., Moussa,M., and Capron,F. (2001). Expression of Epstein-Barr Virus-Induced Gene 3, an Interleukin-12 p40-Related Molecule, throughout Human Pregnancy : Involvement of Syncytiotrophoblasts and Extravillous Trophoblasts. *Am J Pathol* 159, 1763-1776.

Dheda,K., Huggett,J.F., Bustin,S.A., Johnson,M.A., Rook,G., and Zumla,A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 37, 112-119.

DiBrino,M., Parker,K.C., Margulies,D.H., Shiloach,J., Turner,R.V., Biddison,W.E., and Coligan,J.E. (1995). Identification of the peptide binding motif for HLA-B44, one of the most common HLA-B alleles in the Caucasian population. *Biochemistry* 34, 10130-10138.

Diehl,V., Franklin,J., Pfreundschuh,M., Lathan,B., Paulus,U., Hasenclever,D., Tesch,H., Herrmann,R., Dorken,B., Muller-Hermelink,H.K., Duhmke,E., and Loeffler,M. (2003). Standard and increased-dose BEACOPP chemotherapy compared with COPP-ABVD for advanced Hodgkin's disease. *N Engl J Med* 348, 2386-2395.

References

- Dotti,G., Fiocchi,R., Motta,T., Gamba,A., Gotti,E., Gridelli,B., Borleri,G., Manzoni,C., Viero,P., Remuzzi,G., Barbui,T., and Rambaldi,A. (2000). Epstein-Barr virus-negative lymphoproliferate disorders in long-term survivors after heart, kidney, and liver transplant. *Transplantation* 69, 827-833.
- Duggan,D.B., Petroni,G.R., Johnson,J.L., Glick,J.H., Fisher,R.I., Connors,J.M., Canellos,G.P., and Peterson,B.A. (2003). Randomized Comparison of ABVD and MOPP/ABV Hybrid for the Treatment of Advanced Hodgkin's Disease: Report of an Intergroup Trial. *J Clin Oncol* 21, 607-614.
- Dunne,P.J., Faint,J.M., Gudgeon,N.H., Fletcher,J.M., Plunkett,F.J., Soares,M.V., Hislop,A.D., Annels,N.E., Rickinson,A.B., Salmon,M., and Akbar,A.N. (2002). Epstein-Barr virus-specific CD8+ T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential. *Blood* 100, 933-940.
- Dupin,N., Fisher,C., Kellam,P., Ariad,S., Tulliez,M., Franck,N., van Marck,E., Salmon,D., Gorin,I., Escande,J.P., Weiss,R.A., Alitalo,K., and Boshoff,C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc. Natl. Acad. Sci. U. S. A* 96, 4546-4551.
- Duraiswamy,J., Bharadwaj,M., Tellam,J., Connolly,G., Cooper,L., Moss,D., Thomson,S., Yotnda,P., and Khanna,R. (2004). Induction of therapeutic T-cell responses to subdominant tumor-associated viral oncogene after immunization with replication-incompetent polyepitope adenovirus vaccine. *Cancer Res* 64, 1483-1489.
- Duraiswamy,J., Sheritt,M., Thomson,S., Tellam,J., Cooper,L., Connolly,G., Bharadwaj,M., and Khanna,R. (2003). Therapeutic LMP1 polyepitope vaccine for EBV-associated Hodgkin disease and nasopharyngeal carcinoma. *Blood* 101, 3150-3156.
- Duval,A., Raphael,M., Brennetot,C., Poirel,H., Buhard,O., Aubry,A., Martin,A., Krimi,A., Leblond,V., Gabarre,J., Davi,F., Charlotte,F., Berger,F., Gaidano,G., Capello,D., Canioni,D., Bordessoule,D., Feuillard,J., Gaulard,P., Delfau,M.H., Ferlicot,S., Eclache,V., Prevot,S., Guettier,C., Lefevre,P.C., Adotti,F., and Hamelin,R. (2004). The mutator pathway is a feature of immunodeficiency-related lymphomas. *PNAS* 101, 5002-5007.
- Ehlin-Henriksson,B., Gordon,J., and Klein,G. (2003). B-lymphocyte subpopulations are equally susceptible to Epstein-Barr virus infection, irrespective of immunoglobulin isotype expression. *Immunology* 108, 427-430.
- Ekerfelt,C., Ernerudh,J., and Jenmalm,M.C. (2002). Detection of spontaneous and antigen-induced human interleukin-4 responses in vitro: comparison of ELISPOT, a novel ELISA and real-time RT-PCR. *J Immunol Methods* 260, 55-67.
- Eliopoulos,A.G., Stack,M., Dawson,C.W., Kaye,K.M., Hodgkin,L., Sihota,S., Rowe,M., and Young,L.S. (1997). Epstein-Barr virus-encoded LMP1 and CD40 mediate IL-6 production in epithelial cells via an NF-kappaB pathway involving TNF receptor-associated factors. *Oncogene* 14, 2899-2916.

References

- Elmaagacli, A.H., Beelen, D.W., Opalka, B., Seeber, S., and Schaefer, U.W. (2000). The amount of BCR-ABL fusion transcripts detected by the real-time quantitative polymerase chain reaction method in patients with Philadelphia chromosome positive chronic myeloid leukemia correlates with the disease stage. *Annals of Hematology* 79, 424-431.
- Enblad, G., Sandvej, K., Sundstrom, C., Pallesen, G., and Glimelius, B. (1999). Epstein-Barr virus distribution in Hodgkin's disease in an unselected Swedish population. *Acta Oncol.* 38, 425-429.
- Epstein, MA, Achong, BG, and Barr, YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* 1, 702-703. 1964.
Ref Type: Abstract
- Euvrard, S., Kanitakis, J., and Claudy, A. (2003). Skin Cancers after Organ Transplantation. *The New England Journal Of Medicine* 348, 1681-1691.
- Falco, D.A., Nepomuceno, R.R., Krams, S.M., Lee, P.P., Davis, M.M., Salvatierra, O., Alexander, S.R., Esquivel, C.O., Cox, K.L., Frankel, L.R., and Martinez, O.M. (2002). Identification of Epstein-Barr virus-specific CD8+ T lymphocytes in the circulation of pediatric transplant recipients. *Transplantation* 74, 501-510.
- Faye, A., Quartier, P., Reguerre, Y., Lutz, P., Carret, A.S., Dehee, A., Rohrlach, P., Peuchmaur, M., Matthieu-Boue, A., Fischer, A., and Vilmer, E. (2001). Chimaeric anti-CD20 monoclonal antibody (rituximab) in post-transplant B-lymphoproliferative disorder following stem cell transplantation in children. *British Journal of Haematology* 115, 112-118.
- Fingerroth, J.D., Clabby, M.L., and Strominger, J.D. (1988). Characterization of a T-lymphocyte Epstein-Barr virus/C3d receptor (CD21). *J Virol.* 62, 1442-1447.
- Fleisher, G., Henle, W., Henle, G., Lennette, E.T., and Biggar, R.J. (1979). Primary infection with Epstein-Barr virus in infants in the United States: clinical and serologic observations. *J Infect. Dis.* 139, 553-558.
- Fleisher, G.R., Pasquariello, P.S., Warren, W.S., Zavod, W.S., Korval, A.B., Turner, H.D., and Lennette, E.T. (1981). Intrafamilial transmission of Epstein-Barr virus infections. *J Pediatr.* 98, 16-19.
- Frisan, T., Sjoberg, J., Dolcetti, R., Boiocchi, M., De Re, V., Carbone, A., Brautbar, C., Battat, S., Biberfeld, P., and Eckman, M. (1995). Local suppression of Epstein-Barr virus (EBV)-specific cytotoxicity in biopsies of EBV-positive Hodgkin's disease. *Blood* 86, 1493-1501.
- Funch, D.P., Walker, A.M., Schneider, G., Ziyadeh, N.J., and Pescovitz, M.D. (2005). Ganciclovir and Acyclovir Reduce the Risk of Post-Transplant Lymphoproliferative Disorder in Renal Transplant Recipients. *American Journal of Transplantation* 5, 2894-2900.
- Gaidano, G., Ballerini, P., Gong, J.Z., Inghirami, G., Neri, A., Newcomb, E.W., Magrath, I.T., Knowles, D.M., and Dalla-Favera, R. (1991). p53 Mutations in Human

References

Lymphoid Malignancies: Association with Burkitt Lymphoma and Chronic Lymphocytic Leukemia. *PNAS* 88, 5413-5417.

Gandhi, M.K., Wills, M.R., Okecha, G., Day, E.K., Hicks, R., Marcus, R.E., Sissons, J.G.P., and Carmichael, A.J. (2003). Late diversification in the clonal composition of human cytomegalovirus-specific CD8⁺ T cells following allogeneic hemopoietic stem cell transplantation. *Blood* 102, 3427-3438.

Garrett, T.J., Chadburn, A., Barr, M.L., Drusin, R.E., Chen, J.M., Schulman, L.L., Smith, C.R., Reison, D.S., Rose, E.A., Michler, R.E., and . (1993). Posttransplantation lymphoproliferative disorders treated with cyclophosphamide-doxorubicin-vincristine-prednisone chemotherapy. *Cancer* 72, 2782-2785.

Gartner, B.C., Schafer, H., Marggraff, K., Eisele, G., Schafer, M., Dilloo, D., Roemer, K., Laws, H.J., Sester, M., Sester, U., Einsele, H., and Mueller-Lantzsch, N. (2002). Evaluation of use of Epstein-Barr viral load in patients after allogeneic stem cell transplantation to diagnose and monitor posttransplant lymphoproliferative disease. *J. Clin. Microbiol.* 40, 351-358.

Gavioli, R., Campos-Lima, P.O., Kurilla, M.G., Kieff, E., Klein, G., and Masucci, M.G. (1992). Recognition of the Epstein-Barr virus-encoded nuclear antigens EBNA-4 and EBNA-6 by HLA-A11-restricted cytotoxic T lymphocytes: implications for down-regulation of HLA-A11 in Burkitt lymphoma. *Proc. Natl. Acad. Sci. U. S. A* 89, 5862-5866.

Ghobrial, I.M., Habermann, T.M., Maurer, M.J., Geyer, S.M., Ristow, K.M., Larson, T.S., Walker, R.C., Ansell, S.M., Macon, W.R., Gores, G.G., Stegall, M.D., and McGregor, C.G. (2005). Prognostic Analysis for Survival in Adult Solid Organ Transplant Recipients With Post-Transplantation Lymphoproliferative Disorders. *J Clin Oncol* 23, 7574-7582.

Gillespie, G.M., Wills, M.R., Appay, V., O'Callaghan, C., Murphy, M., Smith, N., Sissons, P., Rowland-Jones, S., Bell, J.I., and Moss, P.A. (2000). Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *J Virol* 74, 8140-8150.

Glenn, M., Rainbow, L., Aurade, F., Davison, A., and Schulz, T.F. (1999). Identification of a Spliced Gene from Kaposi's Sarcoma-Associated Herpesvirus Encoding a Protein with Similarities to Latent Membrane Proteins 1 and 2A of Epstein-Barr Virus. *J. Virol.* 73, 6953-6963.

Gold, M.C., Munks, M.W., Wagner, M., Koszinowski, U.H., Hill, A.B., and Fling, S.P. (2002). The murine cytomegalovirus immunomodulatory gene m152 prevents recognition of infected cells by M45-specific CTL but does not alter the immunodominance of the M45-specific CD8 T cell response in vivo. *Journal Of Immunology (Baltimore, Md. : 1950)* 169, 359-365.

Goldsby, R.A., Kindt, T.J., Osbourne, B.A., and Kuby, J. (2003). *Immunology*. (New York: W.H. Freeman).

References

- Grace,M.B., McLeland,C.B., Gagliardi,S.J., Smith,J.M., Jackson,W.E., III, and Blakely,W.F. (2003). Development and Assessment of a Quantitative Reverse Transcription-PCR Assay for Simultaneous Measurement of Four Amplicons. *Clin Chem* 49, 1467-1475.
- Grasser,F.A., Murray,P.G., Kremmer,E., Klein,K., Remberger,K., Feiden,W., Reynolds,G., Niedobitek,G., Young,L.S., and Mueller-Lantzsch,N. (1994). Monoclonal antibodies directed against the Epstein-Barr virus-encoded nuclear antigen 1 (EBNA1): immunohistologic detection of EBNA1 in the malignant cells of Hodgkin's disease. *Blood* 84, 3792-3798.
- Gratama,J.W., Oosterveer,M.A., Klein,G., and Ernberg,I. (1990). EBNA size polymorphism can be used to trace Epstein-Barr virus spread within families. *J Virol* 64, 4703-4708.
- Green,M., Bueno,J., Rowe,D., Mazariegos,G., Qu,L., bu-Almagd,K., and Reyes,J. (2000). Predictive negative value of persistent low Epstein-Barr virus viral load after intestinal transplantation in children. *Transplantation* 70, 593-596.
- Green,M., Cacciarelli,T.V., Mazariegos,G.V., Sigurdsson,L., Qu,L., Rowe,D.T., and Reyes,J. (1998). Serial measurement of Epstein-Barr viral load in peripheral blood in pediatric liver transplant recipients during treatment for posttransplant lymphoproliferative disease. *Transplantation*. 66, 1641-1644.
- Green,M., Kaufmann,M., Wilson,J., and Reyes,J. (1997). Comparison of intravenous ganciclovir followed by oral acyclovir with intravenous ganciclovir alone for prevention of cytomegalovirus and Epstein-Barr virus disease after liver transplantation in children. *Clin Infect Dis* 25, 1344-1349.
- Greenwood,B.M. and Vick,R.M. (1975). Evidence for a malaria mitogen in human malaria. *Nature* 257, 592-594.
- Gregory,C.D., Rowe,M., and Rickinson,A.B. (1990). Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. *J Gen. Virol.* 71 (Pt 7), 1481-1495.
- Gridelli,B., Spada,M., Riva,S., Colledan,M., Segalin,A., Lucianetti,A., Sonzogni,A., Furione,M., Baldanti,F., and Torre,G. (2000). Circulating Epstein-Barr virus DNA to monitor lymphoproliferative disease following pediatric liver transplantation. *Transpl. Int.* 13 Suppl 1, S399-S401.
- Gross,T.G., Hinrichs,S.H., Winner,J., Greiner,T.C., Kaufman,S.S., Sammut,P.H., and Langnas,A.N. (1998). Treatment of post-transplant lymphoproliferative disease (PTLD) following solid organ transplantation with low-dose chemotherapy. *Ann Oncol* 9, 339-340.
- Gross,T.G., Bucuvalas,J.C., Park,J.R., Greiner,T.C., Hinrich,S.H., Kaufman,S.S., Langnas,A.N., McDonald,R.A., Ryckman,F.C., Shaw,B.W., Sudan,D.L., and Lynch,J.C. (2005). Low-Dose Chemotherapy for Epstein-Barr Virus-Positive Post-Transplantation Lymphoproliferative Disease in Children After Solid Organ Transplantation. *J Clin Oncol* 23, 6481-6488.

References

- Grossman,S.R., Johannsen,E., Tong,X., Yalamanchili,R., and Kieff,E. (1994). The Epstein-Barr Virus Nuclear Antigen 2 Transactivator is Directed to Response Elements by the J{kappa} Recombination Signal Binding Protein. *PNAS* 91, 7568-7572.
- Gu,S.Y., Huang,T.M., Ruan,L., Miao,Y.H., Lu,H., Chu,C.M., Motz,M., and Wolf,H. (1995). First EBV vaccine trial in humans using recombinant vaccinia virus expressing the major membrane antigen. *Dev. Biol. Stand.* 84, 171-177.
- Guihot,A., Dupin,N., Marcelin,A.G., Gorin,I., Bedin,A.S., Bossi,P., Galicier,L., Oksenhendler,E., Autran,B., and Carcelain,G. (2006). Low T Cell Responses to Human Herpesvirus 8 in Patients with AIDS-Related and Classic Kaposi Sarcoma. *The Journal Of Infectious Diseases* 194, 1078-1088.
- Gustafsson,A., Levitsky,V., Zou,J.Z., Frisan,T., Dalianis,T., Ljungman,P., Ringden,O., Winiarski,J., Ernberg,I., and Masucci,M.G. (2000). Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. *Blood* 95, 807-814.
- Gutensohn,N. and Cole,P. (1980). Epidemiology of Hodgkin's disease. *Semin. Oncol* 7, 92-102.
- Haddad,E., Paczesny,S., Leblond,V., Seigneurin,J.M., Stern,M., Achkar,A., Bauwens,M., Delwail,V., Debray,D., Duvoux,C., Hubert,P., de Ligny,B.H., Wijdenes,J., Durandy,A., and Fischer,A. (2001). Treatment of B-lymphoproliferative disorder with a monoclonal anti-interleukin-6 antibody in 12 patients: a multicenter phase 1-2 clinical trial. *Blood* 97, 1590-1597.
- Hale,G., Waldmann,H., and CAMPATH,U. (1998). Risks of Developing Epstein-Barr Virus-Related Lymphoproliferative Disorders After T-Cell-Depleted Marrow Transplants. *Blood* 91, 3079-3083.
- Hallee,T.J., Evans,A.S., Niederman,J.C., Brooks,C.M., and Voegtly,j.H. (1974). Infectious mononucleosis at the United States Military Academy. A prospective study of a single class over four years. *The Yale Journal Of Biology And Medicine* 47, 182-195.
- Hamilton,J.K., Paquin,L.A., Sullivan,J.L., Maurer,H.S., Cruzei,F.G., Provisor,A.J., Steuber,C.P., Hawkins,E., Yawn,D., Cornet,J.A., Clausen,K., Finkelstein,G.Z., Landing,B., Grunnet,M., and Purtilo,D.T. (1980). X-linked lymphoproliferative syndrome registry report. *J Pediatr.* 96, 669-673.
- Haque,T., Taylor,C., Wilkie,G.M., Murad,P., Amlot,P.L., Beath,S., McKiernan,P.J., and Crawford,D.H. (2001). Complete regression of posttransplant lymphoproliferative disease using partially HLA-matched Epstein Barr virus-specific cytotoxic T cells. *Transplantation* 72, 1399-1402.
- Haque,T., Wilkie,G.M., Taylor,C., Amlot,P.L., Murad,P., Iley,A., Dombagoda,D., Britton,K.M., Swerdlow,A.J., and Crawford,D.H. (2002). Treatment of Epstein-

References

- Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells. *Lancet* 360, 436-442.
- Harabuchi, Y., Yamanaka, N., Kataura, A., Imai, S., Kinoshita, T., Mizuno, F., and Osato, T. (1990). Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma. *Lancet* 335, 128-130.
- Harari, A., Vallelleian, F., Meylan, P.R., and Pantaleo, G. (2005). Functional Heterogeneity of Memory CD4 T Cell Responses in Different Conditions of Antigen Exposure and Persistence. *J Immunol* 174, 1037-1045.
- Harris, N.L., Jaffe, E.S., Stein, H., Banks, P.M., Chan, J.K., Cleary, M.L., Delsol, G., Wolf-Peters, C., Falini, B., and Gatter et, a. (1994). A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 84, 1361-1392.
- Harris, P.E., Colovai, A., Liu, Z., Dalla, F.R., and Suci-Foca, N. (1993). Naturally processed HLA class I bound peptides from c-myc-transfected cells reveal allele-specific motifs. *J Immunol* 151, 5966-5974.
- Hartmann, F., Renner, C., Jung, W., da Costa, L., Tembrink, S., Held, G., Sek, A., Konig, J., Bauer, S., Kloft, M., and Pfreundschuh, M. (2001). Anti-CD16/CD30 Bispecific Antibody Treatment for Hodgkin's Disease: Role of Infusion Schedule and Costimulation with Cytokines. *Clin Cancer Res* 7, 1873-1881.
- Hasenclever, D. and Diehl, V. (1998). A prognostic score for advanced Hodgkin's disease. International Prognostic Factors Project on Advanced Hodgkin's Disease. *N Engl J Med* 339, 1506-1514.
- Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein-Barr Virus-Coded BHRF1 Protein, a Viral Homologue of Bcl-2, Protects Human B Cells from Programmed Cell Death. *PNAS* 90, 8479-8483.
- Herbst, H., Foss, H.D., Samol, J., Araujo, I., Klotzbach, H., Krause, H., Agathangelou, A., Niedobitek, G., and Stein, H. (1996). Frequent expression of interleukin-10 by Epstein-Barr virus-harboring tumor cells of Hodgkin's disease. *Blood* 87, 2918-2929.
- Hill, A.B., Lee, S.P., Haurum, J.S., Murray, N., Yao, Q.Y., Rowe, M., Signore, N., Rickinson, A.B., and McMichael, A.J. (1995). Class I major histocompatibility complex-restricted cytotoxic T lymphocytes specific for Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines against which they were raised. *J Exp. Med.* 181, 2221-2228.
- Hislop, A.D., Gudgeon, N.H., Callan, M.F., Fazou, C., Hasegawa, H., Salmon, M., and Rickinson, A.B. (2001). EBV-specific CD8+ T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. *J Immunol.* 167, 2019-2029.
- Hislop, A.D., Annels, N.E., Gudgeon, N.H., Leese, A.M., and Rickinson, A.B. (2002). Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J. Exp. Med.* 195, 893-905.

References

- Ho,M., Jaffe,R., Miller,G., Breinig,M.K., Dummer,J.S., Makowka,L., Atchison,R.W., Karrer,F., Nalesnik,M.A., and Starzl,T.E. (1988). The frequency of Epstein-Barr virus infection and associated lymphoproliferative syndrome after transplantation and its manifestations in children. *Transplantation* 45, 719-727.
- Hochberg,D.R. and Thorley-Lawson,D.A. (2005b). Quantitative detection of viral gene expression in populations of Epstein-Barr virus-infected cells in vivo. *Methods Mol Biol.* 292, 39-56.
- Hochberg,D.R. and Thorley-Lawson,D.A. (2005a). Quantitative detection of viral gene expression in populations of Epstein-Barr virus-infected cells in vivo. *Methods Mol Biol.* 292, 39-56.
- Hollsberg,P. (2002). Contribution of HLA class I allele expression to CD8+ T-cell responses against Epstein-Barr virus. *Scand. J Immunol.* 55, 189-195.
- Holmes,R.D., Orban-Eller,K., Karrer,F.R., Rowe,D.T., Narkewicz,M.R., and Sokol,R.J. (2002). Response of elevated Epstein-Barr virus DNA levels to therapeutic changes in pediatric liver transplant patients: 56-month follow up and outcome. *Transplantation* 74, 367-372.
- Horning,S.J., Hoppe,R.T., Breslin,S., Bartlett,N.L., Brown,B.W., and Rosenberg,S.A. (2002). Stanford V and radiotherapy for locally extensive and advanced Hodgkin's disease: mature results of a prospective clinical trial. *J Clin Oncol* 20, 630-637.
- Hoshino,Y., Morishima,T., Kimura,H., Nishikawa,K., Tsurumi,T., and Kuzushima,K. (1999). Antigen-driven expansion and contraction of CD8+-activated T cells in primary EBV infection. *J Immunol.* 163, 5735-5740.
- Hosler,G.A., Bash,R.O., Bai,X., Jain,V., and Scheuermann,R.H. (1999). Development and Validation of a Quantitative Polymerase Chain Reaction Assay to Evaluate Minimal Residual Disease for T-Cell Acute Lymphoblastic Leukemia and Follicular Lymphoma. *Am J Pathol* 154, 1023-1035.
- Howe,J.G. and Steitz,J.A. (1986). Localization of Epstein-Barr Virus-Encoded Small RNAs by in situ Hybridization. *PNAS* 83, 9006-9010.
- Howie,D., Sayos,J., Terhorst,C., and Morra,M. (2000). The gene defective in X-linked lymphoproliferative disease controls T cell dependent immune surveillance against Epstein-Barr virus. *Curr. Opin. Immunol* 12, 474-478.
- Hsieh,J.J. and Hayward,S.D. (1995). Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* 268, 560-563.
- Hsu,D.H., de Waal,M.R., Fiorentino,D.F., Dang,M.N., Vieira,P., de Vries,J., Spits,H., Mosmann,T.R., and Moore,K.W. (1990). Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. *Science* 250, 830-832.

References

- Hsu,S.M., Lin,J., Xie,S.S., Hsu,P.L., and Rich,S. (1993). Abundant expression of transforming growth factor-beta 1 and -beta 2 by Hodgkin's Reed-Sternberg cells and by reactive T lymphocytes in Hodgkin's disease. *Hum. Pathol* 24, 249-255.
- Hu,H., Tang,K.F., Chua,Y.N., Lu,J., Feng,P., Chew,C.T., and Chan,S.H. (2004). Expression of interleukin-18 by nasopharyngeal carcinoma cells: a factor that possibly initiates the massive leukocyte infiltration. *Human Pathology* 35, 722-728.
- Huang,Y.T., Sheen,T.S., Chen,C.L., Lu,J., Chang,Y., Chen,J.Y., and Tsai,C.H. (1999). Profile of Cytokine Expression in Nasopharyngeal Carcinomas: A Distinct Expression of Interleukin 1 in Tumor and CD4+ T Cells. *Cancer Res* 59, 1599-1605.
- Imai,S., Koizumi,S., Sugiura,M., Tokunaga,M., Uemura,Y., Yamamoto,N., Tanaka,S., Sato,E., and Osato,T. (1994). Gastric Carcinoma: Monoclonal Epithelial Malignant Cells Expressing Epstein-Barr Virus Latent Infection Protein. *PNAS* 91, 9131-9135.
- Imai,S., Nishikawa,J., and Takada,K. (1998). Cell-to-Cell Contact as an Efficient Mode of Epstein-Barr Virus Infection of Diverse Human Epithelial Cells. *J. Virol.* 72, 4371-4378.
- Israele,V., Shirley,P., and Sixbey,J.W. (1991). Excretion of the Epstein-Barr virus from the genital tract of men. *The Journal Of Infectious Diseases* 163, 1341-1343.
- Jaffe,E.S., Harris,N.L., Stein,H., and Vardiman,J.W. (2001). World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. (Lyon, France: IARC Press), pp. 264-271.
- Jarrett,R.F. (2002). Viruses and Hodgkin's lymphoma. *Annals of Oncology* 13, 23-29.
- Jarrett,R.F., Stark,G.L., White,J., Angus,B., Alexander,F.E., Krajewski,A.S., Freeland,J., Taylor,G.M., and Taylor,P.R. (2005). Impact of tumor Epstein-Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma: a population-based study. *Blood* 106, 2444-2451.
- Johannessen,I., Asghar,M., and Crawford,D.H. (2000). Essential role for T cells in human B-cell lymphoproliferative disease development in severe combined immunodeficient mice. *British Journal of Haematology* 109, 600-610.
- Jones,J.F., Shurin,S., Abramowsky,C., Tubbs,R.R., Sciotto,C.G., Wahl,R., Sands,J., Gottman,D., Katz,B.Z., and Sklar,J. (1988). T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. *N. Engl. J Med.* 318, 733-741.
- Joseph,A.M., Babcock,G.J., and Thorley-Lawson,D.A. (2000). Cells expressing the Epstein-Barr virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils. *J Virol.* 74, 9964-9971.
- Kammula,U.S., Lee,K.H., Riker,A.I., Wang,E., Ohnmacht,G.A., Rosenberg,S.A., and Marincola,F.M. (1999). Functional analysis of antigen-specific T lymphocytes

by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol.* 163, 6867-6875.

Kammula,U.S., Marincola,F.M., and Rosenberg,S.A. (2000). Real-Time Quantitative Polymerase Chain Reaction Assessment of Immune Reactivity in Melanoma Patients After Tumor Peptide Vaccination. *J Natl Cancer Inst* 92, 1336-1344.

Kanavaros,P., Lescs,M.C., Briere,J., Divine,M., Galateau,F., Joab,I., Bosq,J., Farcet,J.P., Reyes,F., and Gaulard,P. (1993). Nasal T-cell lymphoma: a clinicopathologic entity associated with peculiar phenotype and with Epstein-Barr virus. *Blood* 81, 2688-2695.

Kanegane,H., Nomura,K., Miyawaki,T., and Tosato,G. (2002). Biological aspects of Epstein-Barr virus (EBV)-infected lymphocytes in chronic active EBV infection and associated malignancies. *Crit Rev. Oncol Hematol.* 44, 239-249.

Kanzler,H., Kuppers,R., Hansmann,M.L., and Rajewsky,K. (1996). Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. *J Exp. Med* 184, 1495-1505.

Karge,W.H., III, Schaefer,E.J., and Ordovas,J.M. (1998). Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. *Methods Mol Biol.* 110, 43-61.

Karnofsky,D.A. and Burchenal,J.H. (1949). The clinical evaluation of chemotherapeutic agents in cancer. In *Evaluation of Chemotherapeutic Agents*, C.M.MacLeod, ed. (New York, NY: Columbia University Press), pp. 199-205.

Karrer,E.E., Lincoln,J.E., Hogenhout,S., Bennett,A.B., Bostock,R.M., Martineau,B., Lucas,W.J., Gilchrist,D.G., and Alexander,D. (1995). In situ Isolation of mRNA from Individual Plant Cells: Creation of Cell-Specific cDNA Libraries. *PNAS* 92, 3814-3818.

Keegan,T.H., Glaser,S.L., Clarke,C.A., Gulley,M.L., Craig,F.E., Digiuseppe,J.A., Dorfman,R.F., Mann,R.B., and Ambinder,R.F. (2005b). Epstein-Barr virus as a marker of survival after Hodgkin's lymphoma: a population-based study. *J. Clin. Oncol.* 23, 7604-7613.

Keegan,T.H., Glaser,S.L., Clarke,C.A., Gulley,M.L., Craig,F.E., Digiuseppe,J.A., Dorfman,R.F., Mann,R.B., and Ambinder,R.F. (2005a). Epstein-Barr virus as a marker of survival after Hodgkin's lymphoma: a population-based study. *J. Clin. Oncol.* 23, 7604-7613.

Kelly,G., Bell,A., and Rickinson,A. (2002). Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. *Nat Med* 8, 1098-1104.

Kennedy,G.A., Tey,S.K., Cobcroft,R., Marlton,P., Cull,G., Grimmett,K., Thomson,D., and Gill,D. (2002). Incidence and nature of CD20-negative relapses

following rituximab therapy in aggressive B-cell non-Hodgkin's lymphoma: a retrospective review. *Br J Haematol.* 119, 412-416.

Kern, F., LiPira, G., Gratama, J.W., Manca, F., and Roederer, M. (2005). Measuring Ag-specific immune responses: understanding immunopathogenesis and improving diagnostics in infectious disease, autoimmunity and cancer. *Trends Immunol.* 26, 477-484.

Khan, N., Hislop, A., Gudgeon, N., Cobbold, M., Khanna, R., Nayak, L., Rickinson, A.B., and Moss, P.A.H. (2004). Herpesvirus-Specific CD8 T Cell Immunity in Old Age: Cytomegalovirus Impairs the Response to a Coresident EBV Infection. *J Immunol* 173, 7481-7489.

Khanna, R., Burrows, S.R., Kurilla, M.G., Jacob, C.A., Misko, I.S., Sculley, T.B., Kieff, E., and Moss, D.J. (1992). Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J Exp. Med.* 176, 169-176.

Khanna, R., Sherritt, M., and Burrows, S.R. (1999). EBV structural antigens, gp350 and gp85, as targets for ex vivo virus-specific CTL during acute infectious mononucleosis: potential use of gp350/gp85 CTL epitopes for vaccine design. *J Immunol.* 162, 3063-3069.

Kienzle, N., Buck, M., Greco, S., Krauer, K., and Sculley, T.B. (1999). Epstein-Barr Virus-Encoded RK-BARF0 Protein Expression. *J. Virol.* 73, 8902-8906.

Kiepiela, P., Leslie, A.J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., Rathnavalu, P., Moore, C., Pfafferoth, K.J., Hilton, L., Zimbwa, P., Moore, S., Allen, T., Brander, C., Addo, M.M., Altfeld, M., James, I., Mallal, S., Bunce, M., Barber, L.D., Szinger, J., Day, C., Klenerman, P., Mullins, J., Korber, B., Coovadia, H.M., Walker, B.D., and Goulder, P.J. (2004). Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* 432, 769-775.

Kimura, H., Morishima, T., Kanegane, H., Ohga, S., Hoshino, Y., Maeda, A., Imai, S., Okano, M., Morio, T., Yokota, S., Tsuchiya, S., Yachie, A., Imashuku, S., Kawa, K., and Wakiguchi, H. (2003). Prognostic factors for chronic active Epstein-Barr virus infection. *J Infect. Dis.* 187, 527-533.

Kimura, H., Hoshino, Y., Kanegane, H., Tsuge, I., Okamura, T., Kawa, K., and Morishima, T. (2001). Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 98, 280-286.

Kis, L.L., Takahara, M., Nagy, N., Klein, G., and Klein, E. (2006). Cytokine mediated induction of the major Epstein-Barr virus (EBV)-encoded transforming protein, LMP-1. *Immunology Letters* 104, 83-88.

Kitagawa, N., Goto, M., Kurozumi, K., Maruo, S., Fukayama, M., Naoe, T., Yasukawa, M., Hino, K., Suzuki, T., Todo, S., and Takada, K. (2000). Epstein-Barr virus-encoded poly(A)(-) RNA supports Burkitt's lymphoma growth through interleukin-10 induction. *EMBO J* 19, 6742-6750.

References

- Klein,G. (1989). Viral latency and transformation: the strategy of Epstein-Barr virus. *Cell* 58, 5-8.
- Klenerman,P., Cerundolo,V., and Dunbar,P.R. (2002). TRACKING T CELLS WITH TETRAMERS: NEW TALES FROM NEW TOOLS. *Nat Rev Immunol* 2, 263-272.
- Kliche,S., Kremmer,E., Hammerschmidt,W., Koszinowski,U., and Haas,J. (1998). Persistent Infection of Epstein-Barr Virus-Positive B Lymphocytes by Human Herpesvirus 8. *J. Virol.* 72, 8143-8149.
- Knowles,D.M., Cesarman,E., Chadburn,A., Frizzera,G., Chen,J., Rose,E.A., and Michler,R.E. (1995). Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders. *Blood* 85, 552-565.
- Knox,P.G., Li,Q.X., Rickinson,A.B., and Young,L.S. (1996). In vitro production of stable Epstein-Barr virus-positive epithelial cell clones which resemble the virus:cell interaction observed in nasopharyngeal carcinoma. *Virology* 215, 40-50.
- Koffman,B.H., Kennedy,A.S., Heyman,M., Colonna,J., and Howell,C. (2000). Use of radiation therapy in posttransplant lymphoproliferative disorder (PTLD) after liver transplantation. *Int. J Cancer* 90, 104-109.
- Kruse,N., Moriabadi,N.F., Toyka,K.V., and Rieckmann,P. (2001). Characterization of early immunological responses in primary cultures of differentially activated human peripheral mononuclear cells. *J Immunol Methods* 247, 131-139.
- Kuehnle,I., Huls,M.H., Liu,Z., Semmelmann,M., Krance,R.A., Brenner,M.K., Rooney,C.M., and Heslop,H.E. (2000). CD20 monoclonal antibody (rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoietic stem-cell transplantation. *Blood* 95, 1502-1505.
- Kullberg-Lindh,C., Ascher,H., Saalman,R., Olausson,M., and Lindh,M. (2006). Epstein-Barr viremia levels after pediatric liver transplantation as measured by real-time polymerase chain reaction. *Pediatr. Transplant.* 10, 83-89.
- Kuppers,R. (2002). Molecular biology of Hodgkin's lymphoma. *Adv. Cancer Res* 84, 277-312.
- Kurth,J., Spieker,T., Wustrow,J., Strickler,G.J., Hansmann,L.M., Rajewsky,K., and Kuppers,R. (2000). EBV-infected B cells in infectious mononucleosis: viral strategies for spreading in the B cell compartment and establishing latency. *Immunity.* 13, 485-495.
- Kurth,J., Hansmann,M.L., Rajewsky,K., and Kuppers,R. (2003). Epstein-Barr virus-infected B cells expanding in germinal centers of infectious mononucleosis patients do not participate in the germinal center reaction. *PNAS* 100, 4730-4735.
- Kuzushima,K., Hoshino,Y., Fujii,K., Yokoyama,N., Fujita,M., Kiyono,T., Kimura,H., Morishima,T., Morishima,Y., and Tsurumi,T. (1999). Rapid

determination of Epstein-Barr virus-specific CD8(+) T-cell frequencies by flow cytometry. *Blood* 94, 3094-3100.

Kuzushima,K., Kimura,H., Hoshino,Y., Yoshimi,A., Tsuge,I., Horibe,K., Morishima,T., Tsurumi,T., and Kojima,S. (2000). Longitudinal dynamics of Epstein-Barr virus-specific cytotoxic T lymphocytes during posttransplant lymphoproliferative disorder. *J Infect Dis* 182, 937-940.

Lacey,S.F., Gallez-Hawkins,G., Crooks,M., Martinez,J., Senitzer,D., Forman,S.J., Spielberger,R., Zaia,J.A., and Diamond,D.J. (2002). Characterization of cytotoxic function of CMV-pp65-specific CD8+ T-lymphocytes identified by HLA tetramers in recipients and donors of stem-cell transplants. *Transplantation* 74, 722-732.

Lacey,S.F., Villacres,M.C., La Rosa,C., Wang,Z., Longmate,J., Martinez,J., Brewer,J.C., Mekhoubad,S., Maas,R., and Leedom,J.M. (2003). Relative dominance of HLA-B*07 restricted CD8+ T-Lymphocyte immune responses to human cytomegalovirus pp65 in persons sharing HLA-A*02 and HLA-B*07 alleles. *Human Immunology* 64, 440-452.

Ladetto,M., Sametti,S., Donovan,J.W., Ferrero,D., Astolfi,M., Mitterer,M., Ricca,I., Drandi,D., Corradini,P., Coser,P., Pileri,A., Gribben,J.G., and Tarella,C. (2001). A validated real-time quantitative PCR approach shows a correlation between tumor burden and successful ex vivo purging in follicular lymphoma patients. *Exp. Hematol.* 29, 183-193.

Laherty,C.D., Hu,H.M., Oipari,A.W., Wang,F., and Dixit,V.M. (1992). The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. *J. Biol. Chem.* 267, 24157-24160.

Lalvani,A., Brookes,R., Hambleton,S., Britton,W.J., Hill,A.V., and McMichael,A.J. (1997). Rapid effector function in CD8+ memory T cells. *J Exp. Med.* 186, 859-865.

Landais,E., Saulquin,X., and Houssaint,E. (2005). The human T cell immune response to Epstein-Barr virus. *Int. J Dev. Biol.* 49, 285-292.

Landay,A.L. (2004). Performance of Single Cell Immune Response Assays; Proposed Guideline. NCCLS document I/LA26-A (ISBN 1-56238-546-1)..

Lane,H.C., Masur,H., Edgar,L.C., Whalen,G., Rook,A.H., and Fauci,A.S. (1983). Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N. Engl. J Med* 309, 453-458.

Lavu,E., Morewaya,J., Maraka,R., Kiromat,M., Ripa,P., and Vince,J. (2005). Burkitt lymphoma in Papua New Guinea--40 years on. *Ann. Trop. Paediatr.* 25, 191-197.

Leaver,S., Amlot,P., Thuraishingam,R., Norton,A., Aitken,C., and Cavenagh,J.D. (2004). Subacute immune response to primary EBV infection leading to post-transplant lymphoproliferative disease in a renal transplant patient. *Clinical and Laboratory Haematology* 26, 351-353.

Leblond,V., Davi,F., Charlotte,F., Dorent,R., Bitker,M.O., Sutton,L., Gandjbakhch,I., Binet,J.L., and Raphael,M. (1998). Posttransplant

lymphoproliferative disorders not associated with Epstein-Barr virus: a distinct entity? *J. Clin. Oncol.* *16*, 2052-2059.

Leblond, V., Dhedin, N., Mamzer Bruneel, M.F., Choquet, S., Hermine, O., Porcher, R., Nguyen, Q.S., Davi, F., Charlotte, F., Dorent, R., Barrou, B., Vernant, J.P., Raphael, M., and Levy, V. (2001). Identification of prognostic factors in 61 patients with posttransplantation lymphoproliferative disorders. *J Clin. Oncol.* *19*, 772-778.

Lechner, F., Gruener, N.H., Urbani, S., Uggeri, J., Santantonio, T., Kammer, A.R., Cerny, A., Phillips, R., Ferrari, C., Pape, G.R., and Klenerman, P. (2000). CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur. J Immunol.* *30*, 2479-2487.

Lee, P.P., Yee, C., Savage, P.A., Fong, L., Brockstedt, D., Weber, J.S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P.D., Roederer, M., and Davis, M.M. (1999). Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med.* *5*, 677-685.

Lee, S.P., Brooks, J.M., Al Jarrah, H., Thomas, W.A., Haigh, T.A., Taylor, G.S., Humme, S., Schepers, A., Hammerschmidt, W., Yates, J.L., Rickinson, A.B., and Blake, N.W. (2004). CD8 T cell recognition of endogenously expressed Epstein-Barr virus nuclear antigen 1. *J Exp. Med.* *199*, 1409-1420.

Lee, T.C., Savoldo, B., Rooney, C.M., Heslop, H.E., Gee, A.P., Caldwell, Y., Barshes, N.R., Scott, J.D., Bristow, L.J., O'Mahony, C.A., and Goss, J.A. (2005). Quantitative EBV viral loads and immunosuppression alterations can decrease PTLTD incidence in pediatric liver transplant recipients. *Am. J. Transplant.* *5*, 2222-2228.

Lefort, K. and Dotto, G.P. (2004). Notch signaling in the integrated control of keratinocyte growth/differentiation and tumor suppression. *Semin. Cancer Biol.* *14*, 374-386.

Leung, E., Shenton, B.K., Green, K., Jackson, G., Gould, F.K., Yap, C., and Talbot, D. (2004). Dynamic EBV gene loads in renal, hepatic, and cardiothoracic transplant recipients as determined by real-time PCR light cycler. *Transpl. Infect. Dis.* *6*, 156-164.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P.M., Klein, G., Kurilla, M.G., and Masucci, M.G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* *375*, 685-688.

Levitskaya, J., Sharipo, A., Leonchiks, A., Ciechanover, A., and Masucci, M.G. (1997). Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *PNAS* *94*, 12616-12621.

Levitsky, V., Liu, D., Southwood, S., Levitskaya, J., Sette, A., and Masucci, M.G. (2000). Supermotif peptide binding and degeneracy of MHC: peptide recognition in an EBV peptide-specific CTL response with highly restricted TCR usage. *Hum. Immunol.* *61*, 972-984.

References

- Li,Q., Spriggs,M.K., Kovats,S., Turk,S.M., Comeau,M.R., Nepom,B., and Hutt-Fletcher,L.M. (1997). Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. *J. Virol.* 71, 4657-4662.
- Libertiny,G., Watson,C.J., Gray,D.W., Welsh,K.I., and Morris,P.J. (2001). Rising incidence of post-transplant lymphoproliferative disease in kidney transplant recipients. *Br J Surg.* 88, 1330-1334.
- Linch,D.C., Winfield,D., Goldstone,A.H., Moir,D., Hancock,B., McMillan,A., Chopra,R., Milligan,D., and Hudson,G.V. (1993). Dose intensification with autologous bone-marrow transplantation in relapsed and resistant Hodgkin's disease: results of a BNLI randomised trial. *Lancet* 341, 1051-1054.
- Listvanova,S., Temmerman,S., Stordeur,P., Verscheure,V., Place,S., Zhou,L., Loch,C., and Mascart,F. (2003). Optimal kinetics for quantification of antigen-induced cytokines in human peripheral blood mononuclear cells by real-time PCR and by ELISA. *Journal of Immunological Methods* 281, 27-35.
- Livak,K.J. and Schmittgen,T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.
- Livingston,B.D., Crimi,C., Fikes,J., Chesnut,R.W., Sidney,J., and Sette,A. (1999). Immunization with the HBV core 18-27 epitope elicits CTL responses in humans expressing different HLA-A2 supertype molecules. *Hum. Immunol* 60, 1013-1017.
- Lo,K.W. and Huang,D.P. (2002). Genetic and epigenetic changes in nasopharyngeal carcinoma. *Semin. Cancer Biol.* 12, 451-462.
- Loeffler,M., Brosteanu,O., Hasenclever,D., Sextro,M., Assouline,D., Bartolucci,A.A., Cassileth,P.A., Crowther,D., Diehl,V., Fisher,R.I., Hoppe,R.T., Jacobs,P., Pater,J.L., Pavlovsky,S., Thompson,E., and Wiernik,P. (1998). Meta-analysis of chemotherapy versus combined modality treatment trials in Hodgkin's disease. International Database on Hodgkin's Disease Overview Study Group. *J Clin Oncol* 16, 818-829.
- Lu,Q.L., Elia,G., Lucas,S., and Thomas,J.A. (1993). Bcl-2 proto-oncogene expression in Epstein-Barr-virus-associated nasopharyngeal carcinoma. *Int. J Cancer* 53, 29-35.
- Lucas,K.G., Salzman,D., Garcia,A., and Sun,Q. (2004). Adoptive immunotherapy with allogeneic Epstein-Barr virus (EBV)-specific cytotoxic T-lymphocytes for recurrent, EBV-positive Hodgkin disease. *Cancer* 100, 1892-1901.
- Magrath,I. (1990). The pathogenesis of Burkitt's lymphoma. *Adv. Cancer Res.* 55, 133-270.
- Maini,M.K., Gudgeon,N., Wedderburn,L.R., Rickinson,A.B., and Beverley,P.C.L. (2000). Clonal Expansions in Acute EBV Infection Are Detectable in the CD8 and not the CD4 Subset and Persist with a Variable CD45 Phenotype. *J Immunol* 165, 5729-5737.

References

- Malec,M., Soderqvist,M., Sirsjo,A., MacNamara,B., Lewin,N., Sjoberg,J., Bjorkholm,M., and Porwit-MacDonald,A. (2004). Real-time polymerase chain reaction determination of cytokine mRNA expression profiles in Hodgkin's lymphoma. *Haematologica* 89, 679-685.
- Marafioti,T., Hummel,M., Foss,H.D., Laumen,H., Korbjuhn,P., Anagnostopoulos,I., Lammert,H., Demel,G., Theil,J., Wirth,T., and Stein,H. (2000). Hodgkin and reed-sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. *Blood* 95, 1443-1450.
- Marcucci,G., Livak,K.J., Bi,W., Strout,M.P., Bloomfield,C.D., and Caligiuri,M.A. (1998). Detection of minimal residual disease in patients with AML1/ETO-associated acute myeloid leukemia using a novel quantitative reverse transcription polymerase chain reaction assay. *Leukemia* 12, 1482-1489.
- Marshall,N.A., Christie,L.E., Munro,L.R., Culligan,D.J., Johnston,P.W., Barker,R.N., and Vickers,M.A. (2004). Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 103, 1755-1762.
- Masucci,M.G., Torsteindottir,S., Colombani,J., Brautbar,C., Klein,E., and Klein,G. (1987). Down-regulation of class I HLA antigens and of the Epstein-Barr virus-encoded latent membrane protein in Burkitt lymphoma lines. *Proc. Natl. Acad. Sci. U. S. A* 84, 4567-4571.
- McClain,K.L., Leach,C.T., Jenson,H.B., Joshi,V.V., Pollock,B.H., Parmley,R.T., DiCarlo,F.J., Chadwick,E.G., and Murphy,S.B. (1995). Association of Epstein-Barr Virus with Leiomyosarcomas in Young People with AIDS. *The New England Journal Of Medicine* 332, 12-18.
- McDiarmid,S.V., Jordan,S., Lee,G.S., Toyoda,M., Goss,J.A., Vargas,J.H., Martin,M.G., Bahar,R., Maxfield,A.L., Ament,M.E., and Busuttil,R.W. (1998). Prevention and preemptive therapy of postransplant lymphoproliferative disease in pediatric liver recipients. *Transplantation* 66, 1604-1611.
- McGuirk,J.P., Seropian,S., Howe,G., Smith,B., Stoddart,L., and Cooper,D.L. (1999). Use of rituximab and irradiated donor-derived lymphocytes to control Epstein-Barr virus-associated lymphoproliferation in patients undergoing related haplo-identical stem cell transplantation. *Bone Marrow Transplant.* 24, 1253-1258.
- Merlino,C., Cavallo,R., Bergallo,M., Giacchino,F., Bollero,C., Negro,P.A., and Cavallo,G. (2003). Epstein Barr viral load monitoring by quantitative PCR in renal transplant patients. *New Microbiol.* 26, 141-149.
- Meru,N., Davison,S., Whitehead,L., Jung,A., Mutimer,D., Rooney,N., Kelly,D., and Niedobitek,G. (2001). Epstein-Barr virus infection in paediatric liver transplant recipients: detection of the virus in post-transplant tonsillectomy specimens. *Mol Pathol* 54, 264-269.

References

- Micheletti, F., Monini, P., Fortini, C., Rimessi, P., Bazzaro, M., Andreoni, M., Giuliani, M., Traniello, S., Ensoli, B., and Gavioli, R. (2002). Identification of cytotoxic T lymphocyte epitopes of human herpesvirus 8. *Immunology* 106, 395-403.
- Miller, C.L., Burkhardt, A.L., Lee, J.H., Stealey, B., Longnecker, R., Bolen, J.B., and Kieff, E. (1995). Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases. *Immunity* 2, 155-166.
- Milpied, N., Vasseur, B., Parquet, N., Garnier, J.L., Antoine, C., Quartier, P., Carret, A.S., Bouscary, D., Faye, A., Bourbigot, B., Reguerre, Y., Stoppa, A.M., Bourquard, P., Hurault, d.L., Dubief, F., Mathieu-Boue, A., and Leblond, V. (2000). Humanized anti-CD20 monoclonal antibody (Rituximab) in post transplant B-lymphoproliferative disorder: a retrospective analysis on 32 patients. *Ann Oncol* 11 Suppl 1, 113-116.
- Mocellin, S., Ohnmacht, G.A., Wang, E., and Marincola, F.M. (2001). Kinetics of cytokine expression in melanoma metastases classifies immune responsiveness. *Int. J Cancer* 93, 236-242.
- Mocellin, S., Provenzano, M., Rossi, C.R., Pilati, P., Nitti, D., and Lise, M. (2003). Use of quantitative real-time PCR to determine immune cell density and cytokine gene profile in the tumor microenvironment. *Journal of Immunological Methods* 280, 1-11.
- Moghaddam, A., Rosenzweig, M., Lee-Parritz, D., Annis, B., Johnson, R.P., and Wang, F. (1997). An animal model for acute and persistent Epstein-Barr virus infection. *Science* 276, 2030-2033.
- Molldrem, J.J., Lee, P.P., Wang, C., Felio, K., Kantarjian, H.M., Champlin, R.E., and Davis, M.M. (2000). Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat. Med.* 6, 1018-1023.
- Morrison, L.A., Lukacher, A.E., Braciale, V.L., Fan, D.P., and Braciale, T.J. (1986). Differences in antigen presentation to MHC class I- and class II- restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163, 903-921.
- Morrison, T.B., Weis, J.J., and Wittwer, C.T. (1998). Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 24, 954-8, 960, 962.
- Mueller, S.N., Jones, C.M., Smith, C.M., Heath, W.R., and Carbone, F.R. (2002). Rapid Cytotoxic T Lymphocyte Activation Occurs in the Draining Lymph Nodes After Cutaneous Herpes Simplex Virus Infection as a Result of Early Antigen Presentation and Not the Presence of Virus. *J. Exp. Med.* 195, 651-656.
- Murray, R.J., Kurilla, M.G., Brooks, J.M., Thomas, W.A., Rowe, M., Kieff, E., and Rickinson, A.B. (1992). Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. *J Exp. Med.* 176, 157-168.

References

- Muti,G., Cantoni,S., Oreste,P., Klersy,C., Gini,G., Rossi,V., D'Avanzo,G., Comoli,P., Baldanti,F., Montillo,M., Nosari,A., and Morra,E. (2002). Post-transplant lymphoproliferative disorders: improved outcome after clinico-pathologically tailored treatment. *Haematologica* 87, 67-77.
- Nakajima,H., Cella,M., Bouchon,A., Grierson,H.L., Lewis,J., Duckett,C.S., Cohen,J.I., and Colonna,M. (2000). Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur. J Immunol* 30, 3309-3318.
- Nanbo,A., Inoue,K., Adachi-Takasawa,K., and Takada,K. (2002). Epstein-Barr virus RNA confers resistance to interferon-alpha-induced apoptosis in Burkitt's lymphoma. *EMBO J* 21, 954-965.
- Nelson,B.P., Nalesnik,M.A., Bahler,D.W., Locker,J., Fung,J.J., and Swerdlow,S.H. (2000). Epstein-Barr virus-negative post-transplant lymphoproliferative disorders: a distinct entity? *Am. J. Surg. Pathol.* 24, 375-385.
- Nemerow,G.R., Wolfert,R., McNaughton,M.E., and Cooper,N.R. (1985). Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). *J Virol.* 55, 347-351.
- Nichols,K.E., Harkin,D.P., Levitz,S., Krainer,M., Kolquist,K.A., Genovese,C., Bernard,A., Ferguson,M., Zuo,L., Snyder,E., Buckler,A.J., Wise,C., Ashley,J., Lovett,M., Valentine,M.B., Look,A.T., Gerald,W., Housman,D.E., and Haber,D.A. (1998). Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *PNAS* 95, 13765-13770.
- Niederman,J.C., Evans,A.S., Subrahmanyam,L., and McCollum,R.W. (1970). Prevalence, incidence and persistence of EB virus antibody in young adults. *The New England Journal Of Medicine* 282, 361-365.
- Niederman,J.C., Miller,G., Pearson,H.A., Pagano,J.S., and Dowaliby,J.M. (1976). Infectious mononucleosis. Epstein-Barr-virus shedding in saliva and the oropharynx. *N. Engl. J Med.* 294, 1355-1359.
- Niedobitek,G., Herbst,H., Young,L.S., Brooks,L., Masucci,M.G., Crocker,J., Rickinson,A.B., and Stein,H. (1992). Patterns of Epstein-Barr virus infection in non-neoplastic lymphoid tissue. *Blood* 79, 2520-2526.
- Niedobitek,G., Pazolt,D., Teichmann,M., and Devergne,O. (2002). Frequent expression of the Epstein-Barr virus (EBV)-induced gene, EB13, an IL-12 p40-related cytokine, in Hodgkin and Reed-Sternberg cells. *J. Pathol.* 198, 310-316.
- Nielsen,M.B., Monsurro,V., Migueles,S.A., Wang,E., Perez-Diez,A., Lee,K.H., Kammula,U., Rosenberg,S.A., and Marincola,F.M. (2000). Status of Activation of Circulating Vaccine-Elicited CD8+ T Cells. *J Immunol* 165, 2287-2296.
- Nikoskelainen,J. and Hanninen,P. (1975). Antibody response to Epstein-Barr virus in infectious mononucleosis. *Infect. Immun.* 11, 42-51.

References

- Niller,H.H., Salamon,D., Ilg,K., Koroknai,A., Banati,F., Bauml,G., Rucker,O., Schwarzmann,F., Wolf,H., and Minarovits,J. (2003). The in vivo binding site for oncoprotein c-Myc in the promoter for Epstein-Barr virus (EBV) encoding RNA (EBER) 1 suggests a specific role for EBV in lymphomagenesis. *Med. Sci. Monit.* 9, HY1-HY9.
- Ning,J.P., Yu,M.C., Wang,Q.S., and Henderson,B.E. (1990). Consumption of salted fish and other risk factors for nasopharyngeal carcinoma (NPC) in Tianjin, a low-risk region for NPC in the People's Republic of China. *J Natl Cancer Inst* 82, 291-296.
- Norbury,C.C. and Sigal,L.J. (2003). Cross priming or direct priming: is that really the question? *Current Opinion in Immunology* 15, 82-88.
- Ohnmacht,G.A., Wang,E., Mocellin,S., Abati,A., Filie,A., Fetsch,P., Riker,A.I., Kammula,U.S., Rosenberg,S.A., and Marincola,F.M. (2001). Short-Term Kinetics of Tumor Antigen Expression in Response to Vaccination. *J Immunol* 167, 1809-1820.
- Ohno,S., Luka,J., Falk,L., and Klein,G. (1977). Detection of a nuclear, EBNA-type antigen in apparently EBNA-negative Herpesvirus papio (HVP)-transformed lymphoid lines by the acid-fixed nuclear binding technique. *Int. J Cancer* 20, 941-946.
- Ohshima,K., Suzumiya,J., Akamatu,M., Takeshita,M., and Kikuchi,M. (1995). Human and viral interleukin-10 in Hodgkin's disease, and its influence on CD4+ and CD8+ T lymphocytes. *Int. J Cancer* 62, 5-10.
- Opferman,J.T., Ober,B.T., and Ashton-Rickardt,P.G. (1999). Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science* 283, 1745-1748.
- Orentas,R.J., Schauer,D.W., Jr., Ellis,F.W., Walczak,J., Casper,J.T., and Margolis,D.A. (2003). Monitoring and modulation of Epstein-Barr virus loads in pediatric transplant patients. *Pediatr. Transplant.* 7, 305-314.
- Osman,M., Kubo,T., Gill,J., Neipel,F., Becker,M., Smith,G., Weiss,R., Gazzard,B., Boshoff,C., and Gotch,F. (1999). Identification of Human Herpesvirus 8-Specific Cytotoxic T-Cell Responses. *J. Virol.* 73, 6136-6140.
- Oudejans,J.J., Dukers,D.F., Jiwa,N.M., van Den Brule,A.J., Grasser,F.A., de Bruin,P.C., Horstman,A., Vos,W., van Gorp,J., Middeldorp,J.M., and Meijer,C.J. (1996). Expression of epstein-barr virus encoded nuclear antigen 1 in benign and malignant tissues harbouring EBV. *J Clin Pathol* 49, 897-902.
- Pajic,A., Staeger,M.S., Dudziak,D., Schuhmacher,M., Spitkovsky,D., Eissner,G., Brielmeier,M., Polack,A., and Bornkamm,G.W. (2001). Antagonistic effects of c-myc and Epstein-Barr virus latent genes on the phenotype of human B cells. *Int. J Cancer* 93, 810-816.
- Pantaleo,G., Soudeyins,H., Demarest,J.F., Vaccarezza,M., Graziosi,C., Paolucci,S., Daucher,M., Cohen,O.J., Denis,F., Biddison,W.E., Sekaly,R.P., and Fauci,A.S. (1997). Evidence for rapid disappearance of initially expanded HIV-specific CD8+

References

T cell clones during primary HIV infection. *Proc. Natl Acad. Sci. U. S. A* 94, 9848-9853.

Parker, G.A., Crook, T., Bain, M., Sara, E.A., Farrell, P.J., and Allday, M.J. (1996). Epstein-Barr virus nuclear antigen (EBNA)3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. *Oncogene* 13, 2541-2549.

Pass, H.A., Schwarz, S.L., Wunderlich, J.R., and Rosenberg, S.A. (1998). Immunization of patients with melanoma peptide vaccines: immunologic assessment using the ELISPOT assay. *Cancer J Sci. Am.* 4, 316-323.

Pathmanathan, R., Prasad, U., Sadler, R., Flynn, K., and Raab-Traub, N. (1995). Clonal Proliferations of Cells Infected with Epstein-Barr Virus in Preinvasive Lesions Related to Nasopharyngeal Carcinoma. *The New England Journal Of Medicine* 333, 693-698.

Pawelec, G., Akbar, A., Caruso, C., Solana, R., Grubeck-Loebenstein, B., and Wikby, A. (2005). Human immunosenescence: is it infectious? *Immunol Rev.* 205, 257-268.

Peggs, K.S., Hunter, A., Chopra, R., Parker, A., Mahendra, P., Milligan, D., Craddock, C., Pettengell, R., Dogan, A., Thomson, K.J., Morris, E.C., Hale, G., Waldmann, H., Goldstone, A.H., Linch, D.C., and Mackinnon, S. (2005). Clinical evidence of a graft-versus-Hodgkin's-lymphoma effect after reduced-intensity allogeneic transplantation. *Lancet* 365, 1934-1941.

Peh, S.C., Kim, L.H., and Poppema, S. (2001). TARC, a CC chemokine, is frequently expressed in classic Hodgkin's lymphoma but not in NLP Hodgkin's lymphoma, T-cell-rich B-cell lymphoma, and most cases of anaplastic large cell lymphoma. *Am J Surg. Pathol* 25, 925-929.

Perera, S.M., Thomas, J.A., Burke, M., and Crawford, D.H. (1998). Analysis of the T-cell micro-environment in Epstein-Barr virus-related post-transplantation B lymphoproliferative disease. *J Pathol* 184, 177-184.

Pfaffl, M. and Hageleit, M. (2001). Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. *Biotechnology Letters* 23, 275-282.

Pfaffl, M.W., Tichopad, A., Prgomet, C., and Neuvians, T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper Γ Excel-based tool using pair-wise correlations. *Biotechnology Letters* 26, 509-515.

Piechaczyk, M., Blanchard, J.M., Marty, L., Dani, C., Panabieres, F., El Sabouty, S., Fort, P., and Jeanteur, P. (1984). Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. *Nucleic Acids Res* 12, 6951-6963.

References

- Pope, J.H., Horne, M.K., and Scott, W. (1968). Transformation of foetal human leukocytes in vitro by filtrates of a human leukaemic cell line containing herpes-like virus. *Int. J Cancer* 3, 857-866.
- Powis, S.J., Townsend, A.R.M., Deverson, E.V., Bastin, J., Butcher, G.W., and Howard, J.C. (1991). Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature* 354, 528-531.
- Precopio, M.L., Sullivan, J.L., Willard, C., Somasundaran, M., and Luzuriaga, K. (2003). Differential Kinetics and Specificity of EBV-Specific CD4⁺ and CD8⁺ T Cells During Primary Infection. *J Immunol* 170, 2590-2598.
- Pudney, V.A., Leese, A.M., Rickinson, A.B., and Hislop, A.D. (2005). CD8⁺ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. *J Exp. Med.* 201, 349-360.
- Raab-Traub, N. and Flynn, K. (1986). The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. *Cell* 47, 883-889.
- Razzouk, B.I., Srinivas, S., Sample, C.E., Singh, V., and Sixbey, J.W. (1996). Epstein-Barr Virus DNA recombination and loss in sporadic Burkitt's lymphoma. *J. Infect. Dis.* 173, 529-535.
- Re, D., Kuppers, R., and Diehl, V. (2005). Molecular Pathogenesis of Hodgkin's Lymphoma. *J Clin Oncol* 23, 6379-6386.
- Redchenko, I.V. and Rickinson, A.B. (1999). Accessing Epstein-Barr virus-specific T-cell memory with peptide-loaded dendritic cells. *J Virol* 73, 334-342.
- Rees, L., Thomas, A., and Amlot, P.L. (1998). Disappearance of an Epstein-Barr virus-positive post-transplant plasmacytoma with reduction of immunosuppression. *Lancet* 352, 789.
- Rentzsch, C., Kayser, S., Stumm, S., Watermann, I., Walter, S., Stevanovic, S., Wallwiener, D., and Guckel, B. (2003). Evaluation of Pre-existent Immunity in Patients with Primary Breast Cancer: Molecular and Cellular Assays to Quantify Antigen-Specific T Lymphocytes in Peripheral Blood Mononuclear Cells. *Clin Cancer Res* 9, 4376-4386.
- Rezvani, K., Grube, M., Brenchley, J.M., Sconocchia, G., Fujiwara, H., Price, D.A., Gostick, E., Yamada, K., Melenhorst, J., Childs, R., Hensel, N., Douek, D.C., and Barrett, A.J. (2003). Functional leukemia-associated antigen-specific memory CD8⁺ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. *Blood* 102, 2892-2900.
- Ribechini, E., Fortini, C., Marastoni, M., Traniello, S., Spisani, S., Monini, P., and Gavioli, R. (2006). Identification of CD8⁺ T cell epitopes within lytic antigens of human herpes virus 8. *J Immunol* 176, 923-930.
- Rickinson, A. and Kieff, E. (2001). In Field's virology, D.Knipe and P.Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 2575-2627.

References

- Rickinson,A.B. and Moss,D.J. (1997). Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. *Annu. Rev. Immunol.* *15*, 405-431.
- Ririe,K.M., Rasmussen,R.P., and Wittwer,C.T. (1997). Product Differentiation by Analysis of DNA Melting Curves during the Polymerase Chain Reaction. *Analytical Biochemistry* *245*, 154-160.
- Rooney,C.M., Roskrow,M.A., Suzuki,N., Ng,C.Y., Brenner,M.K., and Heslop,H. (1998a). Treatment of relapsed Hodgkin's disease using EBV-specific cytotoxic T cells. *Ann Oncol* *9 Suppl 5*, S129-S132.
- Rooney,C.M., Smith,C.A., Ng,C.Y., Loftin,S., Li,C., Krance,R.A., Brenner,M.K., and Heslop,H.E. (1995). Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* *345*, 9-13.
- Rooney,C.M., Smith,C.A., Ng,C.Y.C., Loftin,S.K., Sixbey,J.W., Gan,Y., Srivastava,D.K., Bowman,L.C., Krance,R.A., Brenner,M.K., and Heslop,H.E. (1998b). Infusion of Cytotoxic T Cells for the Prevention and Treatment of Epstein-Barr Virus-Induced Lymphoma in Allogeneic Transplant Recipients. *Blood* *92*, 1549-1555.
- Roskrow,M.A., Suzuki,N., Gan,Y.j., Sixbey,J.W., Ng,C.Y.C., Kimbrough,S., Hudson,M., Brenner,M.K., Heslop,H.E., and Rooney,C.M. (1998). Epstein-Barr Virus (EBV)-Specific Cytotoxic T Lymphocytes for the Treatment of Patients With EBV-Positive Relapsed Hodgkin's Disease. *Blood* *91*, 2925-2934.
- Rowlands,D.C., Ito,M., Mangham,D.C., Reynolds,G., Herbst,H., Hallissey,M.T., Fielding,J.W., Newbold,K.M., Jones,E.L., and Young,L.S. (1993). Epstein-Barr virus and carcinomas: rare association of the virus with gastric adenocarcinomas. *Br. J Cancer* *68*, 1014-1019.
- Ruf,I.K., Rhyne,P.W., Yang,C., Cleveland,J.L., and Sample,J.T. (2000). Epstein-Barr Virus Small RNAs Potentiate Tumorigenicity of Burkitt Lymphoma Cells Independently of an Effect on Apoptosis. *J. Virol.* *74*, 10223-10228.
- Ruppert,J., Sidney,J., Celis,E., Kubo,R.T., Grey,H.M., and Sette,A. (1993). Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell* *74*, 929-937.
- Ryan,J.L., Fan,H., Glaser,S.L., Schichman,S.A., Raab-Traub,N., and Gulley,M.L. (2004). Epstein-Barr virus quantitation by real-time PCR targeting multiple gene segments: a novel approach to screen for the virus in paraffin-embedded tissue and plasma. *J Mol Diagn.* *6*, 378-385.
- Santodonato,L., D'Agostino,G., Nisini,R., Mariotti,S., Monque,D.M., Spada,M., Lattanzi,L., Perrone,M.P., Andreotti,M., Belardelli,F., and Ferrantini,M. (2003). Monocyte-Derived Dendritic Cells Generated After a Short-Term Culture with IFN- α and Granulocyte-Macrophage Colony-Stimulating Factor Stimulate a Potent Epstein-Barr Virus-Specific CD8(+) T Cell Response
3. *J. Immunol.* *170*, 5195-5202.

References

- Saulquin,X., Ibisch,C., Peyrat,M.A., Scotet,E., Hourmant,M., Vie,H., Bonneville,M., and Houssaint,E. (2000). A global appraisal of immunodominant CD8 T cell responses to Epstein-Barr virus and cytomegalovirus by bulk screening. *Eur. J Immunol* 30, 2531-2539.
- Savoldo,B., Rooney,C.M., Quiros-Tejeira,R.E., Caldwell,Y., Wagner,H.J., Lee,T., Finegold,M.J., Dotti,G., Heslop,H.E., and Goss,J.A. (2005). Cellular immunity to Epstein-Barr virus in liver transplant recipients treated with rituximab for post-transplant lymphoproliferative disease. *Am. J. Transplant.* 5, 566-572.
- Savoldo,B., Goss,J.A., Hammer,M.M., Zhang,L., Lopez,T., Gee,A.P., Lin,Y.F., Quiros-Tejeira,R.E., Reinke,P., Schubert,S., Gottschalk,S., Finegold,M.J., Brenner,M.K., Rooney,C.M., and Heslop,H.E. (2006). Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood* 108, 2942-2949.
- Sawyer,R.N., Evans,A.S., Niederman,J.C., and McCollum,R.W. (1971). Prospective studies of a group of Yale University freshmen. I. Occurrence of infectious mononucleosis. *The Journal Of Infectious Diseases* 123, 263-270.
- Sayos,J., Wu,C., Morra,M., Wang,N., Zhang,X., Allen,D., van Schaik,S., Notarangelo,L., Geha,R., Roncarolo,M.G., Oettgen,H., De Vries,J.E., Aversa,G., and Terhorst,C. (1998). The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* 395, 462-469.
- Scheenstra,R., Verschuuren,E.A., de,H.A., Slooff,M.J., The,T.H., Bijleveld,C.M., and Verkade,H.J. (2004). The value of prospective monitoring of Epstein-Barr virus DNA in blood samples of pediatric liver transplant recipients. *Transpl. Infect. Dis.* 6, 15-22.
- Schmitz,N., Pfistner,B., Sextro,M., Sieber,M., Carella,A.M., Haenel,M., Boissevain,F., Zschaber,R., Muller,P., Kirchner,H., Lohri,A., Decker,S., Koch,B., Hasenclever,D., Goldstone,A.H., and Diehl,V. (2002). Aggressive conventional chemotherapy compared with high-dose chemotherapy with autologous haemopoietic stem-cell transplantation for relapsed chemosensitive Hodgkin's disease: a randomised trial. *Lancet* 359, 2065-2071.
- Scholle,F., Bendt,K.M., and Raab-Traub,N. (2000). Epstein-Barr Virus LMP2A Transforms Epithelial Cells, Inhibits Cell Differentiation, and Activates Akt. *J. Virol.* 74, 10681-10689.
- Schwering,I., Brauninger,A., Klein,U., Jungnickel,B., Tinguely,M., Diehl,V., Hansmann,M.L., Dalla-Favera,R., Rajewsky,K., and Kuppers,R. (2003). Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. *Blood* 101, 1505-1512.
- Sette,A. and Sidney,J. (1999). Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 50, 201-212.
- Sette,A., Sidney,J., del Guercio,M.F., Southwood,S., Ruppert,J., Dahlberg,C., Grey,H.M., and Kubo,R.T. (1994). Peptide binding to the most frequent HLA-A

References

- class I alleles measured by quantitative molecular binding assays. *Mol Immunol* 31, 813-822.
- Shannon-Lowe, C.D., Neuhierl, B., Baldwin, G., Rickinson, A.B., and Delecluse, H.J. (2006). From the Cover: Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells. *PNAS* 103, 7065-7070.
- Shapiro, R., Nalesnik, M., McCauley, J., Fedorek, S., Jordan, M.L., Scantlebury, V.P., Jain, A., Vivas, C., Ellis, D., Lombardozzi-Lane, S., Randhawa, P., Johnston, J., Hakala, T.R., Simmons, R.L., Fung, J.J., and Starzl, T.E. (1999). Posttransplant lymphoproliferative disorders in adult and pediatric renal transplant patients receiving tacrolimus-based immunosuppression. *Transplantation* 68, 1851-1854.
- Shi, S.R., Key, M.E., and Kalra, K.L. (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem. Cytochem.* 39, 741-748.
- Shibata, D. and Weiss, L.M. (1992). Epstein-Barr virus-associated gastric adenocarcinoma. *Am J Pathol* 140, 769-774.
- Shimizu, N., Tanabe-Tochikura, A., Kuroiwa, Y., and Takada, K. (1994). Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. *J. Virol.* 68, 6069-6073.
- Silins, S.L., Cross, S.M., Elliott, S.L., Pye, S.J., Burrows, S.R., Burrows, J.M., Moss, D.J., Argat, V.P., and Misko, I.S. (1996). Development of Epstein-Barr virus-specific memory T cell receptor clonotypes in acute infectious mononucleosis. *J Exp. Med.* 184, 1815-1824.
- Silins, S.L., Sherritt, M.A., Silleri, J.M., Cross, S.M., Elliott, S.L., Bharadwaj, M., Le, T.T., Morrison, L.E., Khanna, R., Moss, D.J., Suhrbier, A., and Misko, I.S. (2001). Asymptomatic primary Epstein-Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load. *Blood* 98, 3739-3744.
- Sivaraman, P. and Lye, W.C. (2001). Epstein-Barr virus-associated T-cell lymphoma in solid organ transplant recipients. *Biomed. Pharmacother.* 55, 366-368.
- Sixbey, J.W., Nedrud, J.G., Raab-Traub, N., Hanes, R.A., and Pagano, J.S. (1984). Epstein-Barr virus replication in oropharyngeal epithelial cells. *The New England Journal Of Medicine* 310, 1225-1230.
- Sixbey, J., Lemon, S., and Pagano, J. (1986). A second site for Epstein-Barr virus shedding: The uterine cervix. *The Lancet* 328, 1122-1124.
- Skinninger, B.F. and Mak, T.W. (2002). The role of cytokines in classical Hodgkin lymphoma. *Blood* 99, 4283-4297.

References

- Sloan,V.S., Cameron,P., Porter,G., Gammon,M., Amaya,M., Mellins,E., and Zaller,D.M. (1995). Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 375, 802-806.
- Smets,F., Latinne,D., Bazin,H., Reding,R., Otte,J.B., Buts,J.P., and Sokal,E.M. (2002). Ratio between Epstein-Barr viral load and anti-Epstein-Barr virus specific T-cell response as a predictive marker of posttransplant lymphoproliferative disease. *Transplantation* 73, 1603-1610.
- Smith,P.R., de Jesus,O., Turner,D., Hollyoake,M., Karstegl,C.E., Griffin,B.E., Karran,L., Wang,Y., Hayward,S.D., and Farrell,P.J. (2000). Structure and Coding Content of CST (BART) Family RNAs of Epstein-Barr Virus. *J. Virol.* 74, 3082-3092.
- Sokal,E.M., Antunes,H., Beguin,C., Bodeus,M., Wallemacq,P., de Ville,d.G., Reding,R., Janssen,M., Buts,J.P., and Otte,J.B. (1997). Early signs and risk factors for the increased incidence of Epstein-Barr virus-related posttransplant lymphoproliferative diseases in pediatric liver transplant recipients treated with tacrolimus. *Transplantation* 64, 1438-1442.
- Stahlberg,A., Kubista,M., and Pfaffl,M. (2004). Comparison of Reverse Transcriptases in Gene Expression Analysis. *Clin Chem* 50, 1678-1680.
- Starzl,T.E., Nalesnik,M.A., Porter,K.A., Ho,M., Iwatsuki,S., Griffith,B.P., Rosenthal,J.T., Hakala,T.R., Shaw,B.W., Jr., Hardesty,R.L., and . (1984). Reversibility of lymphomas and lymphoproliferative lesions developing under cyclosporin-steroid therapy. *Lancet* 1, 583-587.
- Steven,N.M., Annels,N.E., Kumar,A., Leese,A.M., Kurilla,M.G., and Rickinson,A.B. (1997). Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *J Exp. Med.* 185, 1605-1617.
- Steven,N.M., Leese,A.M., Annels,N.E., Lee,S.P., and Rickinson,A.B. (1996). Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. *J Exp. Med.* 184, 1801-1813.
- Stevens,S.J., Verkuijlen,S.A., and Middeldorp,J.M. (2005). Quantitative detection of Epstein-Barr virus DNA in clinical specimens by rapid real-time PCR targeting a highly conserved region of EBNA-1. *Methods Mol Biol.* 292, 15-26.
- Stordeur,P., Poulin,L.F., Craciun,L., Zhou,L., Schandene,L., de Lavareille,A., Goriely,S., and Goldman,M. (2002). Cytokine mRNA quantification by real-time PCR. *J Immunol Methods* 259, 55-64.
- Stout,J.T., Chen,H.Y., Brennand,J., Caskey,C.T., and Brinster,R.L. (1985). Expression of human HPRT in the central nervous system of transgenic mice. *Nature* 317, 250-252.
- Straathof,K.C., Bollard,C.M., Papat,U., Huls,M.H., Lopez,T., Morriss,M.C., Gresik,M.V., Gee,A.P., Russell,H.V., Brenner,M.K., Rooney,C.M., and Heslop,H.E.

References

- (2005). Treatment of nasopharyngeal carcinoma with Epstein-Barr virus--specific T lymphocytes. *Blood* 105, 1898-1904.
- Subklewe,M., Chahroudi,A., Schmaljohn,A., Kurilla,M.G., Bhardwaj,N., and Steinman,R.M. (1999). Induction of Epstein-Barr virus-specific cytotoxic T-lymphocyte responses using dendritic cells pulsed with EBNA-3A peptides or UV-inactivated, recombinant EBNA-3A vaccinia virus. *Blood* 94, 1372-1381.
- Subklewe,M., Sebelin,K., Block,A., Meier,A., Roukens,A., Paludan,C., Fonteneau,J.F., Steinman,R.M., and Munz,C. (2005). Dendritic cells expand Epstein Barr virus specific CD8+ T cell responses more efficiently than EBV transformed B cells. *Hum. Immunol.* 66, 938-949.
- Sun,R., Lin,S.F., Gradoville,L., Yuan,Y., Zhu,F., and Miller,G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. *PNAS* 95, 10866-10871.
- Suzan,F., Ammor,M., and Ribrag,V. (2001). Fatal reactivation of cytomegalovirus infection after use of rituximab for a post-transplantation lymphoproliferative disorder. *N Engl J Med* 345, 1000.
- Sylwester,A.W., Mitchell,B.L., Edgar,J.B., Taormina,C., Pelte,C., Ruchti,F., Sleath,P.R., Grabstein,K.H., Hosken,N.A., Kern,F., Nelson,J.A., and Picker,L.J. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J. Exp. Med.* 202, 673-685.
- Szekely,L., Selivanova,G., Magnusson,K.P., Klein,G., and Wiman,K.G. (1993). EBNA-5, an Epstein-Barr Virus-Encoded Nuclear Antigen, Binds to the Retinoblastoma and p53 Proteins. *PNAS* 90, 5455-5459.
- Takada,K. (1999). Epstein-Barr virus and gastric carcinoma. *EBV reports* 6, 95-100.
- Takada,K. and Nanbo,A. (2001). The role of EBERs in oncogenesis. *Semin. Cancer Biol.* 11, 461-467.
- Tamaki,H., Beaulieu,B.L., Somasundaran,M., and Sullivan,J.L. (1995). Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses to Epstein-Barr virus in children. *J Infect. Dis.* 172, 739-746.
- Tan,L.C., Gudgeon,N., Annels,N.E., Hansasuta,P., O'Callaghan,C.A., Rowland-Jones,S., McMichael,A.J., Rickinson,A.B., and Callan,M.F.C. (1999). A Re-Evaluation of the Frequency of CD8+ T Cells Specific for EBV in Healthy Virus Carriers. *J Immunol* 162, 1827-1835.
- Tassignon,J., Burny,W., Dahmani,S., Zhou,L., Stordeur,P., Byl,B., and De Groote,D. (2005). Monitoring of cellular responses after vaccination against tetanus toxoid: comparison of the measurement of IFN-gamma production by ELISA, ELISPOT, flow cytometry and real-time PCR. *J Immunol. Methods* 305, 188-198.

References

- Taylor,A.L., Marcus,R., and Bradley,J.A. (2005). Post-transplant lymphoproliferative disorders (PTLD) after solid organ transplantation. *Critical Reviews in Oncology/Hematology* 56, 155-167.
- Tellam,J., Connolly,G., Green,K.J., Miles,J.J., Moss,D.J., Burrows,S.R., and Khanna,R. (2004). Endogenous presentation of CD8+ T cell epitopes from Epstein-Barr virus-encoded nuclear antigen 1. *J Exp. Med.* 199, 1421-1431.
- Therasse,P., Arbuck,S.G., Eisenhauer,E.A., Wanders,J., Kaplan,R.S., Rubinstein,L., Verweij,J., Van Glabbeke,M., van Oosterom,A.T., Christian,M.C., and Gwyther,S.G. (2000). New Guidelines to Evaluate the Response to Treatment in Solid Tumors. *J Natl Cancer Inst* 92, 205-216.
- Thomas,J.A., Crawford,D.H., and Burke,M. (1995). Clinicopathologic implications of Epstein-Barr virus related B cell lymphoma in immunocompromised patients. *J Clin Pathol* 48, 287-290.
- Thomas,J.A., Hotchin,N.A., Allday,M.J., Amlot,P., Rose,M., Yacoub,M., and Crawford,D.H. (1990). Immunohistology of Epstein-Barr virus-associated antigens in B cell disorders from immunocompromised individuals. *Transplantation* 49, 944-953.
- Thompson,M.P. and Kurzrock,R. (2004). Epstein-Barr Virus and Cancer. *Clin Cancer Res* 10, 803-821.
- Thorley-Lawson,D.A., Ianelli,C., Klamman,L.D., Staunton,D., and Yokoyama,S. (1993). Function of CD48 and its regulation by Epstein-Barr virus. *Biochem. Soc. Trans.* 21, 976-980.
- Thorpe,C.J. and Travers,P.J. (1994). Prediction of an HLA-B44 binding motif by the alignment of known epitopes and molecular modeling of the antigen binding cleft. *Immunogenetics* 40, 303-305.
- Threlkeld,S.C., Wentworth,P.A., Kalams,S.A., Wilkes,B.M., Ruhl,D.J., Keogh,E., Sidney,J., Southwood,S., Walker,B.D., and Sette,A. (1997). Degenerate and promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily: implications for vaccine development. *J Immunol* 159, 1648-1657.
- Timms,J.M., Bell,A., Flavell,J.R., Murray,P.G., Rickinson,A.B., Traverse-Glehen,A., Berger,F., and Delecluse,H.J. (2003). Target cells of Epstein-Barr-virus (EBV)-positive post-transplant lymphoproliferative disease: similarities to EBV-positive Hodgkin's lymphoma. *Lancet* 361, 217-223.
- Tokunaga,M., Land,C.E., Uemura,Y., Tokudome,T., Tanaka,S., and Sato,E. (1993). Epstein-Barr virus in gastric carcinoma. *Am J Pathol* 143, 1250-1254.
- Torsteinsdottir,S., Brautbar,C., Klein,G., Klein,E., and Masucci,M.G. (1988). Differential expression of HLA antigens on human B-cell lines of normal and malignant origin: a consequence of immune surveillance or a phenotypic vestige of the progenitor cells? *Int. J. Cancer* 41, 913-919.

References

- Trofe, J., Buell, J.F., Beebe, T.M., Hanaway, M.J., First, M.R., Alloway, R.R., Gross, T.G., Succop, P., and Woodle, E.S. (2005). Analysis of Factors that Influence Survival with Post-Transplant Lymphoproliferative Disorder in Renal Transplant Recipients: The Israel Penn International Transplant Tumor Registry Experience. *American Journal of Transplantation* 5, 775-780.
- Tsai, D.E., Hardy, C.L., Tomaszewski, J.E., Kotloff, R.M., Oltoff, K.M., Somer, B.G., Schuster, S.J., Porter, D.L., Montone, K.T., and Stadtmauer, E.A. (2001). Reduction in immunosuppression as initial therapy for posttransplant lymphoproliferative disorder: analysis of prognostic variables and long-term follow-up of 42 adult patients. *Transplantation* 71, 1076-1088.
- Tsuchiyama, J., Yoshino, T., Mori, M., Kondoh, E., Oka, T., Akagi, T., Hiraki, A., Nakayama, H., Shibuya, A., Ma, Y., Kawabata, T., Okada, S., and Harada, M. (1998). Characterization of a novel human natural killer-cell line (NK-YS) established from natural killer cell lymphoma/leukemia associated with Epstein-Barr virus infection. *Blood* 92, 1374-1383.
- Vajro, P., Lucariello, S., Migliaro, F., Sokal, E., Gridelli, B., Vegnente, A., Iorio, R., Smets, F., Quinto, I., and Scala, G. (2000). Predictive value of Epstein-Barr virus genome copy number and BZLF1 expression in blood lymphocytes of transplant recipients at risk for lymphoproliferative disease. *J. Infect. Dis.* 181, 2050-2054.
- van den, B.A., Visser, L., and Poppema, S. (1999). High expression of the CC chemokine TARC in Reed-Sternberg cells. A possible explanation for the characteristic T-cell infiltrate in Hodgkin's lymphoma. *Am J Pathol* 154, 1685-1691.
- van Esser, J.W.J., Niesters, H.G.M., Thijsen, S.F.T., Meijer, E., Osterhaus, A.D.M.E., Wolthers, K.C., Boucher, C.A.B., Gratama, J.W., Budel, L.M., van der Holt, B., van Loon, A.M., Lowenberg, B., Verdonck, L.F., and Cornelissen, J.J. (2001). Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. *British Journal of Haematology* 113, 814-821.
- Vasef, M.A., Ferlito, A., and Weiss, L.M. (1997). Nasopharyngeal carcinoma, with emphasis on its relationship to Epstein-Barr virus. *Ann. Otol. Rhinol. Laryngol.* 106, 348-356.
- Voo, K.S., Fu, T., Wang, H.Y., Tellam, J., Heslop, H.E., Brenner, M.K., Rooney, C.M., and Wang, R.F. (2004). Evidence for the presentation of major histocompatibility complex class I-restricted Epstein-Barr virus nuclear antigen 1 peptides to CD8⁺ T lymphocytes. *J Exp. Med.* 199, 459-470.
- Voo, K.S., Fu, T., Heslop, H.E., Brenner, M.K., Rooney, C.M., and Wang, R.F. (2002). Identification of HLA-DP3-restricted Peptides from EBNA1 Recognized by CD4⁺ T Cells. *Cancer Res* 62, 7195-7199.
- Waldrop, S.L., Davis, K.A., Maino, V.C., and Picker, L.J. (1998). Normal human CD4⁺ memory T cells display broad heterogeneity in their activation threshold for cytokine synthesis. *J. Immunol.* 161, 5284-5295.

References

- Waldrop, S.L., Pitcher, C.J., Peterson, D.M., Maino, V.C., and Picker, L.J. (1997). Determination of antigen-specific memory/effector CD4⁺ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J Clin. Invest* 99, 1739-1750.
- Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A., and Kieff, E. (1990). Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J Virol.* 64, 2309-2318.
- Wang, Q.J., Jenkins, F.J., Jacobson, L.P., Kingsley, L.A., Day, R.D., Zhang, Z.W., Meng, Y.X., Pellet, P.E., Kousoulas, K.G., Baghian, A., and Rinaldo, C.R., Jr. (2001). Primary human herpesvirus 8 infection generates a broadly specific CD8⁺ T-cell response to viral lytic cycle proteins. *Blood* 97, 2366-2373.
- Webster, G.J., Reignat, S., Maini, M.K., Whalley, S.A., Ogg, G.S., King, A., Brown, D., Amlot, P.L., Williams, R., Vergani, D., Dusheiko, G.M., and Bertoletti, A. (2000). Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. *Hepatology* 32, 1117-1124.
- Weinstock, D.M., Ambrossi, G.G., Brennan, C., Kiehn, T.E., and Jakubowski, A. (2006). Preemptive diagnosis and treatment of Epstein-Barr virus-associated post transplant lymphoproliferative disorder after hematopoietic stem cell transplant: an approach in development. *Bone Marrow Transplant.* 37, 539-546.
- Weiss, L.M., Movahed, L.A., Warnke, R.A., and Sklar, J. (1989). Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. *N. Engl. J Med.* 320, 502-506.
- Weiss, L.M., Strickler, J.G., Warnke, R.A., Purtilo, D.T., and Sklar, J. (1987). Epstein-Barr viral DNA in tissues of Hodgkin's disease. *Am J Pathol* 129, 86-91.
- Wilkie, G.M., Taylor, C., Jones, M.M., Burns, D.M., Turner, M., Kilpatrick, D., Amlot, P.L., Crawford, D.H., and Haque, T. (2004). Establishment and characterization of a bank of cytotoxic T lymphocytes for immunotherapy of Epstein-Barr virus-associated diseases. *J Immunother.* 27, 309-316.
- Wilkinson, J., Cope, A., Gill, J., Bourboulia, D., Hayes, P., Imami, N., Kubo, T., Marcelin, A., Calvez, V., Weiss, R., Gazzard, B., Boshoff, C., and Gotch, F. (2002). Identification of Kaposi's sarcoma-associated herpesvirus (KSHV)-specific cytotoxic T-lymphocyte epitopes and evaluation of reconstitution of KSHV-specific responses in human immunodeficiency virus type 1-Infected patients receiving highly active antiretroviral therapy. *J Virol.* 76, 2634-2640.
- Williams, H., Macsween, K., McAulay, K., Higgins, C., Harrison, N., Swerdlow, A., Britton, K., and Crawford, D. (2004). Analysis of immune activation and clinical events in acute infectious mononucleosis. *J Infect. Dis.* 190, 63-71.
- Wilson, J.D., Ogg, G.S., Allen, R.L., Davis, C., Shaunak, S., Downie, J., Dyer, W., Workman, C., Sullivan, S., McMichael, A.J., and Rowland-Jones, S.L. (2000). Direct

References

- visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. *AIDS* 14, 225-233.
- Wood, K.J. and Sawitzki, B. (2006). Interferon [gamma]: a crucial role in the function of induced regulatory T cells in vivo. *Trends in Immunology* 27, 183-187.
- Wu, T.C., Mann, R.B., Charache, P., Hayward, S.D., Staal, S., Lambe, B.C., and Ambinder, R.F. (1990). Detection of EBV gene expression in Reed-Sternberg cells of Hodgkin's disease. *Int. J Cancer* 46, 801-804.
- Yang, J., Lemas, V.M., Flinn, I.W., Krone, C., and Ambinder, R.F. (2000a). Application of the ELISPOT assay to the characterization of CD8(+) responses to Epstein-Barr virus antigens. *Blood* 95, 241-248.
- Yang, J., Tao, Q., Flinn, I.W., Murray, P.G., Post, L.E., Ma, H., Piantadosi, S., Caligiuri, M.A., and Ambinder, R.F. (2000b). Characterization of Epstein-Barr virus-infected B cells in patients with posttransplantation lymphoproliferative disease: disappearance after rituximab therapy does not predict clinical response. *Blood* 96, 4055-4063.
- Yang, S.Y., Milford, E., Hammerling, U., and Dupont, B. (1989). Description of the reference panel of B-lymphoblastoid cell lines for Factors of the HLA System: The B-cell line panel designed for the Tenth International Histocompatibility Workshop. (New York: Springer-Verlag).
- Yao, Q. Y., Rickinson, A.B., and Epstein, M.A. (1985). A re-examination of the Epstein-Barr virus carrier state in healthy seropositive individuals. *Int. J Cancer* 35, 35-42.
- Yates, J.L., Warren, N., and Sugden, B. (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* 313, 812-815.
- Young, L., Alfieri, C., Hennessy, K., Evans, H., O'Hara, C., Anderson, K.C., Ritz, J., Shapiro, R.S., Rickinson, A., Kieff, E., and . (1989). Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N. Engl. J Med.* 321, 1080-1085.
- Young, L.S. and Murray, P.G. (2003). Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene* 22, 5108-5121.
- Yu, M.C. and Yuan, J.M. (2002). Epidemiology of nasopharyngeal carcinoma. *Semin. Cancer Biol.* 12, 421-429.
- Zech, L., Haglund, U., Nilsson, K., and Klein, G. (1976). Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt and non-Burkitt lymphomas. *Int. J Cancer* 17, 47-56.
- Zimber-Strobl, U. and Strobl, L.J. (2001). EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes. *Semin. Cancer Biol.* 11, 423-434.

References

Zompi,S., Tulliez,M., Conti,F., Leblond,V., Gaulard,P., Blanche,P., Durand,F., Ghandi,D., Dreyfus,F., Louvel,A., Calmus,Y., and Bouscary,D. (2000). Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with clonal lymphoproliferative disorders after orthotopic liver transplantation: a report of three cases. *J Hepatol.* 32, 521-527.

zur Hausen,A., Schulte-Holthausen,H., Klein,G., Henle,W., Henle,G., Clifford,P., and Santesson,L. (1970). EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. *Nature* 228, 1056-1058.

zur Hausen,A., van Rees,B.P., van Beek,J., Craanen,M.E., Bloemena,E., Offerhaus,G.J.A., Meijer,C.J.L.M., and van den Brule,A.J.C. (2004). Epstein-Barr virus in gastric carcinomas and gastric stump carcinomas: a late event in gastric carcinogenesis. *J Clin Pathol* 57, 487-491.

